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The Role of Inflammatory Molecules in Hypertension

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THE ROLE OF INFLAMMATORY MOLECULES IN HYPERTENSION

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"Don't worry. As long as you hit that wire with the connecting hook at precisely 88mph the instant the lightning strikes the tower... everything will be fine."

- Dr. Emmett Brown; Back to the Future

On occasion in life, a series of highly unlikely events conspire to bring you to a moment entirely unseen, but a moment, in this case, that brings great joy. Several months ago, much less 6 years ago, I could not have envisioned that I would be typing an acknowledgements section for a Dissertation manuscript comprising only portions of my research to date, and for that, I have many people to thank.

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In conclusion, science, and much like the quote, is a journey, and the people who have supported and helped me to this point, are a series of seemingly unlikely events that came together and produced amazing, if not an improbable result. I feel that the product written herein, as well as the product that is myself, is proof of this very concept. My hypothesis from the first day in lab is correct, I have and will succeed.

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LIST OF ABBREVIATIONS

%FS	% fractional shortening
$\cdot ClO^-$	hypochlorite
H_2O_2	hydrogen peroxide
$\cdot O_2^-$	superoxide
·OH	hydroxyl moiety
$\cdot OH^-$	hydroxyl radical
ONOO·	peroxynitrite
i.m.	intramuscular
i.p.	intraperitoneal
p.o.	orally
s.c.	subcutaneously
ACE2	angiotensin converting enzyme 2
aCSF	artificial cerebrospinal fluid
Ad	adenovirus/adenoviral vector
AdEmpty	adenoviral vector with an empty cassette region (control virus)
ADH	anti-diuretic hormone/vasopressin
AdIκB	adenoviral vector containing an inhibitory-*kappa*B cassette insert
AHA	American Heart Association
Ang (1-7)	angiotensin (1-7)
Ang (1-9)	angiotensin (1-9)
Ang I	angiotensin I
Ang II	angiotensin II

Ang III	angiotensin III
Ang IV	angiotensin IV
ANOVA	analysis of variance
ANP	atrial natriuretic peptide
AP	area postrema
ARB(s)	angiotenis receptor blocker(s)
AT_1R	angiotensin II type-1 receptor
AT_2R	angiotensin II type-2 receptor
AT_4R	angiotensin II type-4 receptor
BBB	blood brain barrier
BH_4	tetrahydrobiopterin
BW	body weight
CDC	Centers for Disease Control
cDNA	complementary DNA
CMH	1-hydroxyl-3-methoxycarbonyl-2, 2, 5, 5-tetramethylpyrrolidine
CNS	central nervous system
CRP	C-reactive protein
Cu/Zn-SOD	copper/zinc SOD
CVD(s)	cardiovascular disease(s)
CVO(s)	circumventricular organ(s)
DHBA	dihydrobetulinic acid
DNA	deoxyribonucleic acid
DOCA	deoxycorticosterone acetate

EDTA	ethylenediaminetetra acetic acid
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
eNOS	endothelial NOS
EPI	epinephrine
EPR	electron paramagnetic spin resonance
ERK1/2	extracellular signal-related kinase 1/2
ESR	electron paramagnetic spin resonance
ETN	etanercept
GABA	*gamma*-amminobutyric acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HAT	histone acetyltransferase
HDAC	histone deacetylase
HDACi	HDAC inhibitor(s)/inhibition
HIF	hypoxia inducible factor
HPLC	high performance liquid chromatography
HR	heart rate
HW	heart weight
HW/BW	heart weight/body weight
HYD	hydralazine
IACUC	Institutional Animal Care and Use Committee
ICV	intracerebroventricular
IL	interleukin

iNOS	inducible NOS
IVSTd	interventricular septal thickness in diastole
IVSTs	interventricular septal thickness in systole
IκB	inhibitory-*kappa*B
KHB	Kreb's HEPES buffer
LV	left ventricle
LVIDd	left ventricular internal diameter in diastole
LVIDs	left ventricular internal diameter in systole
LVPWTd	left ventricular posterior wall thickness in diastole
LVPWTs	left ventricular posterior wall thickness in systole
LW	lung weight
LW/BW	lung weight/body weight
MAP	mean arterial pressure
MAPK	mitogen-activated protein kinase
MasR	*Mas* receptor
MCP-1	monocyte chemotactant protein-1
MEF2	myocyte enhancer factor-2
MHC	myosin heavy chain
MHz	megahertz
mRNA	messenger RNA
NADPH	nicotinamide adenine dinucleotide phosphate
NE	norepinephrine
NEP	neutral endopeptidase

NFκB	nuclear factor-*kappa*B
NIH	National Institutes of Health
nNOS	neuronal NOS
NO	nitric oxide
NOS	nitric oxide synthase
Nox1	NADPH oxidase with gp91*phox* homologue
Nox2	NADPH oxidase with gp91*phox*
Nox4	NADPH oxidase with gp91*phox* homologue
NTS	nucleus tractus solitarius
OVLT	organum vasculosum lamina terminalis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDTC	pyrrolidine dithiocarbamate
PEG-SOD	polyethylene glyco-conjugated SOD
PIC(s)	proinflammatory cytokine(s)
PVN	paraventricular nucleus
RAAS	renin-angiotensin-aldosterone system
RAS	renin-angiotensin system
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-PCR	reverse transcriptase PCR
RVLM	rostral ventrolateral medulla
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SEM	standard error of the mean
SFO	subfornical organ
SHR	spontaneously hypertensive
SHRSP	SHR stroke-prone
siRNA	small interfering RNA
SNS	sympathetic nervous system
SOD	superoxide dismutase
SON	supraoptic nucleus
Tei	(isovolumic contraction time + isovolumic relaxation time)/ejection time
Temp	Tempol
TNF	tumor necrosis factor-*alpha*
TNFR1	TNF receptor type-1
TNFR2	TNF receptor type-2
VLM	ventrolateral medulla
VPA	valproic acid
WHO	World Health Organization
WKY	wistar-kyoto

ABSTRACT

Hypertension is a serious condition with high morbidity and mortality rates, as well as a major predisposing factor for multiple cardiovascular and renal diseases. Angiotensin II (Ang II) of the renin-angiotensin system (RAS) plays a pivotal role in propagating the hypertensive response. Moreover, hypertension is considered a chronic low-grade inflammatory condition that intimately involves the actions of proinflammatory cytokines (PICs) such as tumor necrosis factor-*alpha* (TNF). Recent evidence highlights the role that inflammation, specifically TNF, plays in hypertension and in regulating the RAS, but the understanding as to how inflammation is regulated following Ang II or TNF activation, as well as the resultant consequences, is currently unclear. Based upon the literature and work from our laboratory, we hypothesize that inflammatory regulation in local tissues alters the systemic hypertensive response, possibly through an inflammatory-driven reactive oxygen species (ROS) mechanism. Therefore, we examined the role of inflammatory mediators in the heart and brain in regulating the hypertensive response through the modulation of ROS, the transcription factor Nuclear Factor-*kappa*B (NFκB) and the dysregulation of components of the RAS. To examine this interaction, we conducted a series of *in vivo* experiments designed to better understand these mechanisms. First, we evaluated the effects of TNF infusion on blood pressure response and RAS component expression in the heart. We further explored these inflammatory mechanisms in spontaneously hypertensive rats through the actions of histone deacetylases in maintaining the hypertensive state through inflammatory modulation. Next, we examined the interaction of TNF in the brain in regulating the RAS in the Ang II-induced hypertensive drive. Finally, we examined how NFκB in the hypothalamic paraventricular nucleus contributed to the dysregulation of the RAS and adverse pressure responses in Ang II-induced hypertension. Combined, these studies demonstrate

a functional and signaling dependence between PICs and the RAS in hypertension, potentially through a ROS-mediated mechanism. These findings provide insight into the important signaling pathways involved in hypertension, as well as outline potential future therapeutic targets for the continued fight against this debilitating disease.

CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

HYPERTENSION

The World Health Organization (WHO) lists hypertension as one of the world's great public health problems and the leading cause of death worldwide (2002). Within the United States, the Centers for Disease Control (CDC) and the American Heart Association (AHA) conclude that more than 74 million American adults, or 1 in 3 ages 20 and over, have high blood pressure (Lloyd-Jones, Adams et al. 2010). Less than 78% of these people know that they suffer from hypertension, and of those that are aware, less than 68% are currently being treated. Hypertension is not only a serious condition with its own high morbidity and mortality rates, it is also a major risk factor for multiple cardiovascular diseases (CVDs), including myocardial infarction, stroke, heart failure, atrial fibrillation, aortic dissection and peripheral arterial disease. Moreover, with the rise in extraneous causes for increased blood pressure, including obesity, salt intake, poor lifestyle choices and a growing population of aging individuals, the incidence of hypertension is rising (Lloyd-Jones, Adams et al. 2010). While physicians do not know the cause for more than 90-95% of their hypertensive patients, the condition is easily detectable. However, and even more disturbing, is that of these individuals, more than half do not have their hypertension under control. In 2006 alone, greater than 56,000 people died in the United States from this debilitating condition.

Hypertension is classified as a systolic/diastolic blood pressure of 140/90 mmHg or higher. However, with epidemiological data suggesting significant increases in CVDs developing at lower pressures, the current definition remains contentious. As a result, physicians are becoming increasingly wary of pre-hypertensive conditions and initiating therapeutics more proactively. Hypertension can be further broken down into primary (essential) or secondary (identifiable) hypertension. In primary hypertension, a single reversible cause for the elevated

blood pressure cannot be identified, roughly accounting for the 90-95% of hypertensive cases observed. For the remaining 5-10% of clinical cases, a more distinct cause can be detected, falling under the moniker secondary hypertensive.

Current therapeutics for primary hypertension are multiple and varied, exploiting the many endogenous controlling mechanisms of normal pressure response as well as the pathophysiological hypertensive contributors. Briefly, these therapies can include lifestyle modifications and/or a bevy of pharmacological therapeutics. Lifestyle modifications can include weight reduction, sodium reduction, diet modification, increased physical activity and moderation of alcohol and tobacco consumption, amongst others. Common pharmacological interventions can include any number of drugs alone or in combination, including, but not limited to, diuretics, β-blockers, α-blockers, Ca^{2+} channel blockers, angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARBs) (Izzo, Sica et al. 2008). However, many current therapeutics fail to have a beneficial effect on over 50% of treated individuals, prompting the need for novel therapeutic approaches and a greater understanding of the mechanistic pathways involved in the pathogenesis and maintenance of hypertension.

THE FUNCTION OF THE RENIN-ANGIOTENSIN SYSTEM IN HYPERTENSION

The Renin-Angiotensin System in Normal Physiology. There are many controlling factors for normal blood pressure regulation, including local autoregulation, baroreceptor signaling and the renin-angiotensin-aldosterone system (RAAS, herein referred to as RAS for renin-angiotensin system) (Guyton and Hall 2006). In its classically understood pathway, following a decrease in blood pressure, the RAS becomes activated. A reduced blood pressure decreases glomerular filtration rate, therefore increasing the time that the filtrate can undergo reabsorption, leading to an increased reabsorption of sodium and chloride ions from the proximal tubules. The increased

chloride concentration is detected by the macula densa of the kidneys and causes the release of renin from the juxtaglomerular cells of the afferent artery. Circulating renin cleaves circulating angiotensinogen (produced and released by the liver) into the decapeptide angiotensin I (Ang I), which is subsequently converted by ACE (abundantly synthesized and located within the vasculature of the lungs) into the biologically active octapeptide angiotensin II (Ang II). Ang II is the primary effector peptide of the RAS, eliciting its actions through either Ang II type-1 or type-2 receptors (AT_1R and AT_2R, respectively) (Allen, Zhuo et al. 2000; Lavoie and Sigmund 2003). The AT_1R is the main receptor through which Ang II acts, inducing vasoconstriction, aldosterone secretion, increased sympathetic activation and sodium retention, amongst its many other actions, all intended to raise blood pressure to its homeostatic levels (Allen, Zhuo et al. 2000). Once normalized, these same factors negatively signal back upon themselves to shut off these arterial pressure raising functions. The AT_2R, the less common of the two receptors, is thought to be cardioprotective and display actions opposing those of the AT_1R, but this concept remains poorly understood. Though the following depicts the classical RAS (also see Figure 1.1), recent research has shown that a majority of the tissues within the body have their own local RAS, including the heart, vasculature and brain, and that these tissue systems are capable of producing all the associated components of the classical RAS (Lavoie and Sigmund 2003).

Further complicating the original understanding of the RAS was the identification of a separate RAS axis that exhibited cardioprotective properties and seemed to be a counterbalance to the actions of Ang II. Following its initial discovery in 2000, ACE2 was shown to cleave Ang I into Ang (1-9), which is subsequently converted to Ang (1-7) by ACE (Donoghue, Hsieh et al. 2000; Tipnis, Hooper et al. 2000; Xu, Sriramula et al. 2011). Ang II can also be converted by ACE2 to Ang (1-7) (Vickers, Hales et al. 2002). By acting through the Mas receptor (MasR),

Ang (1-7) has actions that oppose those of Ang II, including vasodilatation and anti-hypertrophic properties (Santos, Simoes e Silva et al. 2003). Additional pathways by which the components of the RAS can act are increasingly coming under investigation. The AT_4R subtype, the roles of Ang III and Ang IV, the chymase activation pathway and a non-AT_1/non-AT_2R are a few of the various novel mechanisms currently being studied (Xu, Sriramula et al. 2011). Regardless, these diverse systems highlight the major difference from our traditional understanding of the RAS to this multifaceted system that is only recently becoming more elucidated.

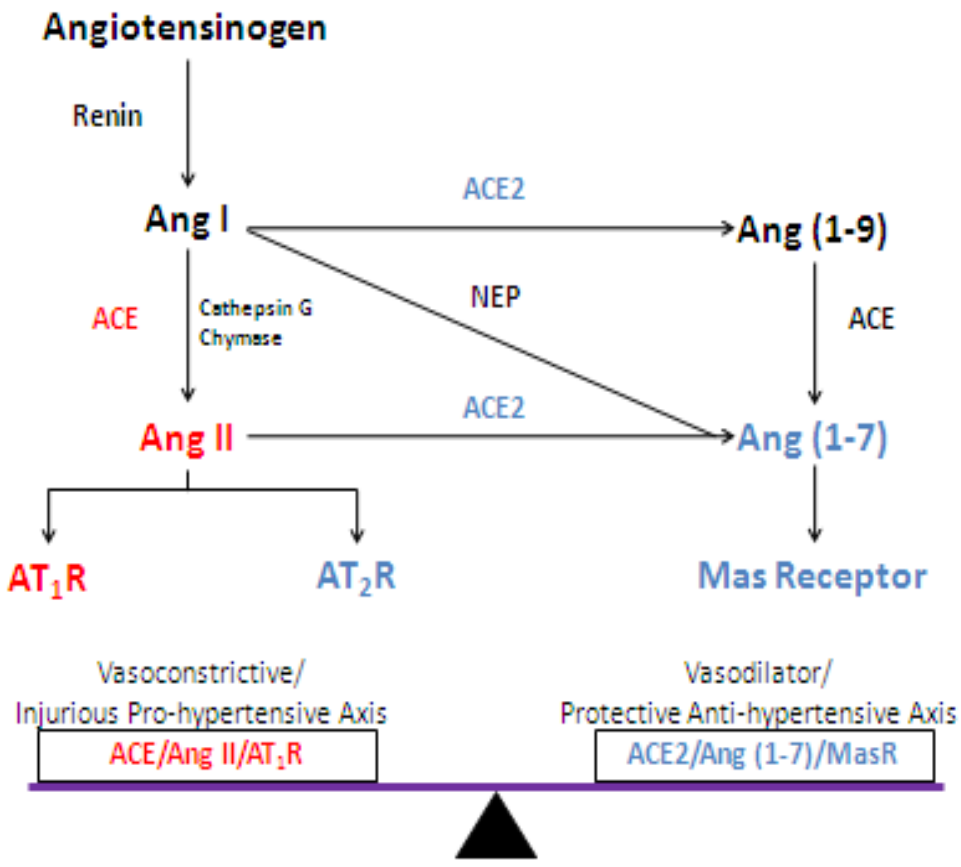

Figure 1.1. The components of the renin-angiotensin system. The balance between the vasoconstrictor/pro-hypertensive and vasodilator/anti-hypertensive axes are critical to the maintenance of blood pressure control and the perpetuation of hypertension. Ang, Angiotensin; ACE, Ang converting enzyme; NEP, Neutral endopeptidase; AT_1 and AT_2R, Ang II Type-1 and Type-2 receptor, respectively.

The RAS in Hypertension. Under typical blood pressure homeostasis, the RAS and its various components function without producing a pathological condition. However, following any of the many triggers that can induce the commencement of a hypertensive response, the RAS becomes intimately involved in its pathogenesis. As such, understanding its function within the hypertensive state is of constant concern. In hypertensive human patients and multiple hypertensive animal models, Ang II is elevated, along with AT_1R expression and increased AT_1R cell surface localization, thereby heightening tissue sensitivity to the already elevated Ang II (Atlas 2007). These tissue responses to Ang II in hypertension are multiple. Initially, a pathological increase in Ang II can lead to sustained elevation of blood pressure through multiple avenues: direct sustained vasoconstriction; increased salt (Na^+) and water retention through direct actions on the kidney tubules, indirectly via aldosterone stimulation and secretion, and/or indirectly via posterior pituitary stimulation and secretion of anti-diuretic hormone (ADH or vasopressin), increased chronic sympathetic activity, etc (Corvol and Jeunemaitre 1997; Ruiz-Ortega, Lorenzo et al. 2001). These factors, combined with additional specific responses of individually activated tissues, exacerbate the hypertensive response and lead to a general deterioration of cardiovascular function and response capabilities.

Over time, sustained elevation of Ang II can also lead to cardiovascular hypertrophy and remodeling, further deteriorating the hypertensive condition (Sasamura, Nakazato et al. 1997; Allen, Zhuo et al. 2000; Paradis, Dali-Youcef et al. 2000; Lim, Lutucuta et al. 2001; Ruiz-Ortega, Lorenzo et al. 2001). Within the heart, Ang II signals cell proliferation and differentiation and cardiomyocyte hypertrophy (Lim, Lutucuta et al. 2001). Additional signaling cascades result in cardiomyocyte contractile dysfunction, interstitial fibrosis, conductance and rhythm defects and a decompensation of cardiac function, which can be reversed by Ang II

receptor blockade (Paradis, Dali-Youcef et al. 2000). This increased remodeling, hypertrophy and heart contractile deficiencies leads to increased peripheral resistance, causes vessel damage, dysfunction of normal vascular mechanisms, and can lead to plaque buildup and atherosclerosis, a major factor for the high morbidity and mortality associated with hypertension (Atlas 2007). Simultaneously, with this increase in the activity of the ACE/Ang II/ AT_1R axis comes the decrease in the protective ACE2/ Ang (1-7)/MasR axis, further reducing the possible cardioprotective and anti-hypertensive effects that this newly recognized arm of the RAS may produce (Xu, Sriramula et al. 2011). Combined, these functions of Ang II are major reasons for the need to control this peptide in hypertension and the rationale behind the use of ACE inhibitors and ARBs in hypertensive therapy (Atlas 2007).

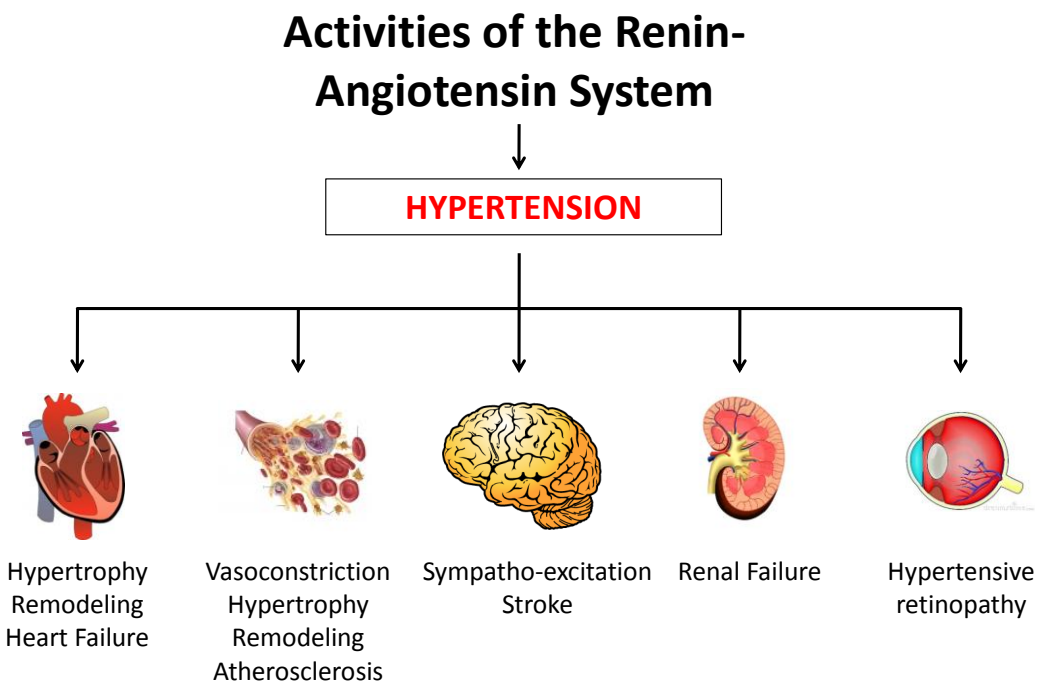

Figure 1.2. Effect of the RAS on organ damage in hypertension. Increased RAS activity is critical in the development and maintenance of hypertension and may lead to end organ damage.

Taken together, the RAS in hypertension is a complicated association of beneficial and deleterious components whose functional interplay is only beginning to come of light. Understanding their functions and the mechanistic pathways by which the RAS is activated and sustained in various tissues in hypertension is instrumental for the advancement of novel therapeutic options, including promoting ACE2 expression and Mas receptor agonists. However, these concepts are intensified in complexity when considering their effect on and their regulation by inflammatory mediators in hypertension.

INFLAMMATION, NUCLEAR FACTOR-κB IN HYPERTENSION

Inflammation is associated with many CVDs, and recent evidence suggests that hypertension is a chronic low-grade inflammatory condition (Ferrario and Strawn 2006; Sriramula, Haque et al. 2008; Kang, Ma et al. 2009; Cardinale, Sriramula et al. 2010). Pro-inflammatory cytokines (PICs) such as Tumor Necrosis Factor-*alpha* (TNF), interleukin (IL)-1β and IL-6, have emerged as major contributing factors in the pathogenesis of hypertension and other CVDs (Mann 2002; Ruiz-Ortega, Ruperez et al. 2002; Sriramula, Haque et al. 2008; Kang, Ma et al. 2009). The RAS, especially the effector peptide Ang II, not only modulates the hypertensive response through the above mentioned mechanisms, but plays a key role in inducing the inflammatory response associated with hypertension (Kalra, Sivasubramanian et al. 2002). Moreover, this inflammatory response appears to upregulate both local and systemic pro-hypertensive components of the RAS while silencing those of the anti-hypertensive axis. The Ang II-mediated inflammatory response can occur through several mechanisms, including activation of monocytes and macrophages, as well as induce the production of adhesion molecules, chemokines, cytokines and ROS activators (Arenas, Xu et al. 2004; Shi, Diez-Freire et al. 2010). While still not fully known, these pathways are becoming more important to

understand, as it is increasingly clear that inflammation plays a major role in the pathophysiological changes observed in the cardiovascular system during sustained Ang II-induced AT_1R activation.

One of the primary cytokines activated by Ang II is TNF. This pleiotrophic peptide exists initially as a membrane bound pre-protein, which once cleaved by metalloproteinases into its soluble form, can elicit its responses through one of two similarly acting TNF receptors, TNFR1 (low affinity) or TNFR2 (high affinity) (Gearing, Beckett et al. 1994; Feldman, Combes et al. 2000; Mann 2002). Once receptor bound, TNF plays an important role in multiple pathophysiological processes, as in hypertension, such as inflammation, cell survival, growth, differentiation and apoptosis (MacEwan 2002). There is much data to support the cross-talk between the RAS and PICs, such as TNF, a conversation often shown to involve the mediation effects of the transcription factor Nuclear Factor-*kappa*B (NFκB) (Mehta and Griendling 2007; Kang, Ma et al. 2009).

NFκB is a key transcription factor regulating many inflammatory and immune responses (Bubici, Papa et al. 2006). This protein signaling cascade is highly complex, with multiple members within the "Rel" and "NFκB" related family of proteins, including RelA (p65), RelB, c-Rel, p50, p52, and additional, less widely distributes peptides, all capable of forming homo- and heterodimers *in vivo* (with the exception of RelB which can only form a homodimer) (Gilmore 2006). These subunits share a Rel-homology domain capable of binding various κB binding sites on target genes and thereby inducing transcription. NFκB is typically sequestered within the cytoplasm of cells and bound to one of several Inhibitory-κB (IκB) proteins such as IκBα, IκBβ or IκBγ, amongst others. Following a signal from any number of possible receptors (within hypertension: via AT_1R, TNFRs and reactive oxygen species (ROS) signaling, etc.), IκB

becomes phosphorylated (typically in hypertension: IκBα), leading to its subsequent ubiquination and degradation. Once freed from IκB, NFκB (often the p50/RelA (p65) heterodimer is observed in hypertension) then translocates itself into the nucleus and binds to its target genes, including additional NFκB and IκB subunits (for negative feedback regulation/transcriptional repression) (Gilmore 2006).

Recent evidence suggests the involvement of NFκB in the pathogenesis of cardiac remodeling and in the failing heart (Kang, Ma et al. 2008; Kang, Ma et al. 2009; Cardinale, Sriramula et al. 2010). Ang II has also been shown to activate NFκB and induce TNF in cardiomyocytes (Cowling, Gurantz et al. 2002). For the hypertension-associated inflammatory response, the p50/p65 dimer promotes gene expression increases for many proteins, including TNF, inducible nitric oxide synthases (iNOSs), and inflammatory adhesion molecules (Sriramula, Haque et al. 2008; Kang, Ma et al. 2009; Cardinale, Sriramula et al. 2010). Activation of NFκB via Ang II not only induces increases in PIC expression, but also leads to increases in ROS production and pro-hypertensive RAS component expression such as AT_1R and ACE, and has also been shown to decrease components of the ACE2/Ang (1-7)/Mas axis. Therefore, understanding NFκB regulation and function within the hypertensive state in inducing the deleterious interactions of the RAS, PICs and ROS is of great interest. Furthermore, in clarifying these concepts, it provides the opportunity to develop novel therapeutic options for controlling the damaging results of hypertension.

REACTIVE OXYGEN SPECIES IN HYPERTENSION

A growing body of evidence indicates that production of ROS and activation of oxidation-reduction dependent signaling cascades leads to oxidative stress and is centrally and critically involved cardiovascular events (Griendling, Sorescu et al. 2000; Touyz 2000; Harrison

and Gongora 2009). ROS and oxidative stress play an important role in diabetes mellitus, atherosclerosis, hyperlipidemia, heart failure, ischemic heart disease and hypertension. Oxidative stress is defined as the imbalanced redox state where pro-oxidants overwhelm intrinsic anti-oxidant systems, resulting in an increased production of ROS. During normal physiological conditions, ROS are produced in a controlled manner at low concentrations and function as signaling molecules or normal by-products of cellular reactions and cellular respiration. Conversely, under pathological conditions, increased ROS production contributes to cell dysfunction, cell growth, monocyte invasion, lipid peroxidation, inflammation, and increased deposition of extracellular matrix proteins, which are all important factors in vascular, renal and cardiac damage during the pathogenesis of hypertension (Sawyer, Siwik et al. 2002; Murdoch, Zhang et al. 2006; Papaharalambus and Griendling 2007).

In mammalian cells, aerobic respiration allows the reduction of molecular oxygen to water. During this process, ROS are generated, including molecular free radical, protein and lipid species. These species are short-lived, and after completing their roles in routine cellular function/maintenance, they are scavenged by a series of antioxidant enzymes (Chabrashvili, Tojo et al. 2002; Majid and Kopkan 2007; Paravicini and Touyz 2008); these highly reactive species however, at elevated and/or uncontrolled concentrations, can interact with and cause damage to proteins, lipids and DNA (Droge 2002; Vaziri 2008). Normally, the *in vivo* anti-oxidant defense is sufficient to metabolize these ROS. However, in conditions of persistent inflammation and oxidative stress, such as hypertension, anti-oxidant molecules and enzymes can be depleted and/or inactivated, thereby impairing the overall anti-oxidant defense system (Droge 2002). Although excessive production of ROS is the most common cause of oxidative stress in hypertension, oxidative stress can also be caused by the primary impairment of antioxidant

systems (Wei and Lee 2002; Ahamed and Siddiqui 2007). Under physiological conditions, ROS produced in the course of normal metabolism are fully reduced by an elaborate cellular and extracellular anti-oxidant defense system. However, in certain pathological conditions like hypertension, increased generation of ROS and/or depletion of anti-oxidant capacity leads to enhanced ROS activity and oxidative stress (Yu 1994; Vaziri, Wang et al. 2000). In fact, several recent studies have demonstrated significant impairment of anti-oxidant enzymes and increased oxidative stress in various models of hypertension, including spontaneously hypertensive (SHR) rats (Carneado, Alvarez de Sotomayor et al. 2002; Elks, Mariappan et al. 2009) and rats with chronic kidney failure (Galle 2001), diabetes (Mariappan, Elks et al. 2010), and Dahl-salt sensitive rats (Wang, Chen et al. 2006; Tian, Moore et al. 2007).

Excessive ROS production causes oxidative damage and is associated with hypertension and other disease conditions (Piotrkowski, Fraga et al. 2007; Puddu, Puddu et al. 2007). Growing evidence from animal and clinical studies suggests that inflammation-induced oxidative stress in the heart and kidney could be a key factor in the development and persistence of hypertension (Cachofeiro, Goicochea et al. ; Vanegas, Ferrebuz et al. 2005; Van Gaal, Mertens et al. 2006). Hypertension itself is a multi-factorial disorder that results from the combined effects of inflammation, ROS, reactive nitrogen species generation, and lipid metabolism. Recent work suggests that cytokines can modulate major ROS molecules, such as superoxide ($\cdot O_2^-$) and nitric oxide (NO), and contribute to hypertensive response (Elks, Mariappan et al. 2008; Sriramula, Haque et al. 2008) and end organ damage (Elks, Mariappan et al. 2008; Mariappan, Elks et al. 2009). Under conditions of excessive ROS production, NO is also depleted, which is often from either a direct decrease in NO (inhibition/depletion of nitric oxide synthase [NOS – converts L-arginine into NO]) or because of a decreased bioavailability of NO due to its rapid interaction

with superoxide to form peroxynitrite (Wilcox 2002; Palm 2006; PechÃ¡novÃ¡, Zicha et al. 2006). At high concentrations, peroxynitrite is also highly toxic to tissues and could be an important signaling molecule and contributor to end organ damage in hypertension.

NO also has many known roles both peripherally and centrally in regulating blood pressure responses. It acts as a vasodilator and neurotransmitter, and it is generally accepted that the level of NO in the brain is inversely proportional to sympathetic outflow (i.e. decreased NO is sympathoexcitatory) (Zucker and Liu 2000; Ramchandra, Barrett et al. 2005). While Ang II, NO, and sympathetic regulation are known to interact, the mechanism is somewhat unclear (Zucker and Liu 2000; Ramchandra, Barrett et al. 2005). Multiple experiments have shown that Ang II infusion both in the periphery and via intracerebroventricular (ICV) cannulation downregulates neuronal NOS (nNOS) in multiple cardio-regulatory regions in the brain, including the paraventricular nucleus (PVN), coinciding with increased sympathetic outflow, and that by scavenging ROS, these changes are reversed (Campese, Shaohua et al. 2005; Tai, Wang et al. 2005). Additionally, in the presence of superoxide, NO is oxidized into peroxynitrite, which further decreases the bioavailability of NO produced by an already diminishing presence of nNOS (Zanzinger 2002). NOS overexpression studies also decreased the hypertensive state in Ang II (Tai, Wang et al. 2005) and SHRSP (Kishi, Hirooka et al. 2003) animal models, highlighting the role that NO plays in sympathetic regulation and blood pressure response. Adding complexity to this system is inducible/inflammatory NOS (iNOS). Activated by Ang II and NFkB (possibly through a redox sensitive mechanism), iNOS overexpression in the rostral ventrolateral medulla (RVLM) causes an increase in sympathetic outflow and mean arterial blood pressure (Kimura, Hirooka et al. 2005). The rationale for this phenomenon lies in the over-efficiency displayed by iNOS compared to the other homologues. By rapidly converting all

available L-arginine to NO, iNOS then begins to produce superoxide and further exasperate the already fragile oxidant state. Thus, this evidence indicates that the increase in sympathoexcitation and continuation of the hypertensive state is, in part, the result of a decreased NO bioavailability in the brain, ultimately due to an increase in oxidative stress.

Although there is evidence that inflammation plays a key role in the pathophysiology of hypertension, it is not clear whether inflammation induces oxidative stress to lead to hypertension or that hypertension induces inflammation and oxidative stress. Regardless the order, or if a combination of both, recent evidence shows that inflammation-induced oxidative stress in hypertension can ultimately lead to heart and kidney failure, as well as an adverse response in the brain and further contributing to the hypertensive response. The imbalance of redox homeostasis is integral to the progression of the hypertensive state.

Sources of ROS. ROS are produced by numerous enzymes in many cell types, including endothelial, vascular smooth muscle, adventitial, neuronal, microglial and various renal cells. The major ROS produced are the superoxide anion ($\cdot O_2^-$), hydroxyl moiety ($\cdot OH$), hypochlorite ($\cdot ClO^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\cdot OH^-$). The superoxide anion can further combine with NO, forming the reactive compound peroxynitrite ($ONOO\cdot$) and generating a nitroso-redox imbalance (Hare and Stamler 2005). Additionally, peroxynitrite oxidizes tetrahydrobiopterin (BH_4), thereby leading to endothelial NOS (eNOS) uncoupling and diminished NO production (Lin, Moran et al. 2005). These ROS are generated as intermediate products in oxidative phosphorylation reactions and play a role in normal redox control of physiological signaling pathways. They also act as important second messengers and intracellular signaling molecules in cell growth, survival and apoptosis. However, excessive ROS generation leads to oxidative stress, triggers cell dysfunction, lipid peroxidation, and DNA

mutagenesis, among multiple additional tissue and cellular mechanistic altering functions (Murdoch, Zhang et al. 2006).

The major source of ROS generation in the cardiovascular system is NADPH oxidase. NADPH oxidase is composed of multiple subunits, including two membrane-bound subunits, p22*phox* and gp91*phox* (also known as Nox2, or the homologues Nox1 and Nox4), and the cytosolic subunits p47*phox*, p40*phox*, p67*phox* and Rac1 (a small G-protein) (Lassegue and Clempus 2003; Lambeth 2004). Upon stimulation, the cytosolic subunits migrate towards the plasma membrane bound subunits and become enzymatically active upon their unification, ultimately yielding superoxide by the 1 electron reduction of oxygen using NADPH as the electron donor: $2O_2+NADPH \rightarrow 2O_2^- + NADP^+$ (Lambeth 2004). Other enzymatic sources of ROS involved are xanthine oxidases, uncoupling of the mitochondrial respiratory chain, cytochrome p450, and uncoupling of eNOS. ROS are physiologically produced, but during diseased states, these sources become the main culprits of sustained cellular and tissue damage, especially during the hypertensive state (Griendling, Sorescu et al. 2000; Harrison and Gongora 2009).

Within the cardiovascular system and during the pathogenesis of hypertension, the balance between the oxidant and anti-oxidant systems tips in favor of the more deleterious oxidative side. If left uncontrolled, it can elicit many changes in the various organ systems required for cardiovascular homeostasis, including the heart, kidney and brain. It is here that over a prolonged period of time, the oxidative unbalance leads to sustained oxidative stress, an exasperated hypertensive response, and eventually, end-organ damage and death.

Redox Balance and Inflammation in Hypertension. The importance of redox signaling mechanisms in the heart, kidneys and brain, and their contribution to end organ damage in hypertension is a relatively new concept. It is of note that activated immune cells release large

quantities of ROS, promoting regional oxidative stress. Oxidative stress may also contribute to hypertensive risk by upregulating production of PICs such as TNF, IL-1β, IL-6, etc. and acute phase proteins such as C-reactive protein (CRP), through activation of the transcription factor NFκB (Barnes and Karin 1997; Himmelfarb 2004), all of which are involved in the hypertensive response. Increased levels of circulating PICs lead to an increase in cytosolic ROS production from sources such as NADPH oxidase. This increase in ROS can signal the mitochondria to increase their ROS production, and can also trigger the phosphorylation of IκBα, which is responsible for maintaining NFκB in its inactive form. Once the IκBα is phosphorylated, NFκB is "activated" and translocates into the nucleus to facilitate the transcription of several PIC geness, including TNF. This gene transcription will lead to further increases in circulating PICs, thereby creating a vicious positive feed-forward cycle.

Not only does ROS activate NFκB, but several lines of *in vitro* experimental evidence indicate that NFκB activation can also lead to ROS generation (Flohe, Brigelius-Flohe et al. 1997; Crack and Taylor 2005). NFκB activation can be triggered by the addition of H_2O_2 to some cell types in the absence of a physiological stimulus, and NFκB activation is inhibited by a broad range of antioxidants (Gloire, Legrand-Poels et al. 2006). Overexpression of copper/zinc superoxide dismutase (CuZn-SOD) enhances NFκB activation by TNF, while overexpression of catalase or of glutathione peroxidase attenuates NFκB activation by TNF (Sakon, Xue et al. 2003; Pham, Bubici et al. 2004; Gloire, Legrand-Poels et al. 2006). There is a bidirectional interaction between oxidative stress and inflammation. H_2O_2 produced from the mitochondria leads to NFκB activation in the cytosol and consequently to the production of PICs. Although the response to oxidative stimuli is short-lived under normal conditions, it can lead to chronic inflammatory responses when the oxidative stimuli are poorly controlled (Chung, Cesari et al.

2009). Furthermore, induction of TNF can increase cellular ROS production, mainly via activation of NADPH oxidase. Due to a positive feed-forward loop, this results in further activation of NFκB and production of TNF (Mariappan, Soorappan et al. 2007; Csiszar, Wang et al. 2008; Mariappan, Elks et al. 2009; Mariappan, Elks et al. 2010). The interaction between oxidative stress and inflammation appears to play an important role in vascular, heart, kidney and brain tissue inflammation and in the development of hypertension. Together, these data indicate that NFκB is also a redox-responsive transcription factor. Thus, the role of inflammation in hypertension cannot be overstated and must be accounted for when addressing the issue of ROS in the pathogenesis of hypertension. Therefore, it is important to understand the PIC and RAS mechanisms contributing to the generation of oxidative stress in hypertension, the effects of this oxidant component of the hypertensive state through PIC and RAS modulation.

THE BRAIN IN HYPERTENSION

Hypertension is a multifaceted disorder involving not only peripheral factors and organ systems, but complex signaling mechanisms into and throughout the central nervous system (CNS) (de Wardener 2001; Guyenet 2006; Peterson, Sharma et al. 2006; Harrison and Gongora 2009; Shi, Raizada et al. 2010). Over the past decade, multiple studies have confirmed the role that central neurogenic mechanisms play in the maintenance and management of blood pressure and volume homeostasis through modulation of the vasculature, heart and kidneys (Guyenet 2006). This includes, but is not limited to, modulation of sympathetic tone, water and salt balance, and system wide regulation of circulatory hormonal release (de Wardener 2001). These nuclei are situated throughout the forebrain, midbrain, and hindbrain regions, each with specific and overlapping roles in cardiovascular homeostasis and response. These nuclei receive inputs

from afferent signals via baro-, chemo-, and osmoreceptors located throughout the body, as well as neural inputs from the circumventricular organs (CVOs), a specialized region of the brain that lacks a fully developed blood brain barrier (BBB) and enables the brain to detect blood-borne signaling hormones and blood osmolality levels through receptors protruding from this area (Guyenet 2006; Peterson, Sharma et al. 2006). These CVOs, along with other cardio-regulatory regions of the brain, are implicated in the maintenance of many experimentally observed forms of hypertension (Harrison and Gongora 2009).

Mounting evidence indicates that the brain plays a major role in the pathogenesis of hypertension and that neurogenic mechanisms are dominant in over 40% of essential hypertensive patients (Esler and Kaye 2003; Hirooka 2008), and more specifically, that the sympathetic nervous system (SNS) plays a major role in the pathogenesis of hypertension (Guyenet 2006; Hirooka 2008). When acutely and chronically activated, the SNS can become involved in 24-hour blood pressure patterns and the sustained progression of hypertension, ultimately resulting in metabolic abnormalities, end-organ damage, and even death (Kishi, Hirooka et al. 2001; Harrison and Gongora 2009). During the past several years, new evidence has also emerged that clearly demonstrates the involvement of PICs, NFκB and ROS in the brain in blood pressure regulation by serving as signaling/modulatory molecules within neurons of cardiovascular regulatory centers and in the regulation of the SNS (Paravicini and Touyz 2006; Peterson, Sharma et al. 2006). These components are linked to sympathetic modulation both during physiological and pathophysiological functions.

The RAS and the Brain. The brain is capable of expressing all the genes that comprise the components of the RAS, including renin, angiotensinogen, Ang I Ang II, Ang (1-7), ACE, ACE2, AT_1R, AT_2R and the Mas receptor (Davisson 2003; Veerasingham and Raizada 2003;

Xu, Sriramula et al. 2011). Recent data indicate that central AT_1R up-regulation plays a critical role in activating pre-sympathetic neurons residing in various cardio-regulatory centers in the brain (Guyenet 2006). The enhancement of neuronal AT_1R transcription appears to be a critical mechanism of this central AT_1R up-regulation. Several factors, including PICs, ROS and RAS signaling affect AT_1R expression and activity, which, in turn, modulates neuronal activity. The pre-sympathetic actions of AT_1Rs in CVD ultimately lead to enhanced sympathoexcitation and controls many cardio-regulatory functions (Ganong 2000). AT_1Rs are located directly on the neurons, and when excited, drive post-sympathetic norepinephrine release to elicit cardiovascular alterations (i.e. increased vasoconstriction, heart rate, renin release, etc.) (Davisson 2003).

The PVN and Hypertension. Within the brain, studies have demonstrated the location of AT_1Rs on many regions associated with systemic and cardiovascular homeostasis. It should also be noted that several regions show a more pronounced effect of receptor activation in regulating short- and long-term sympathetic regulation, including, but not limited to, the CVOs, the hypothalamic PVN and the ventrolateral medulla (VLM) (Davisson 2003; Coote 2005). The CVOs directly detect the presence of Ang II through AT_1R activation, thus serving as a major entry point for the Ang II signal into the brain. The PVN appears to be the main integrative site of the many cardiovascular inputs to the CNS; it also has a large number of AT_1Rs located throughout its region (Dampney 1994; Francis, Weiss et al. 2001; Coote 2005; Kang, Ma et al. 2009). This site has direct projections to the pituitary gland and can increase ADH secretion, as well as neurons that project to the adrenal glands and elicit aldosterone synthesis and secretion (Coote 2005). Finally, the VLM is the main exit point of sympathetic neurons from the brain to the spinal column and into the periphery, also receiving signals from the PVN (Dampney 1994). Taken together, activation of AT_1Rs can induce systemic pressor effects via the sympathetic

nervous system, can blunt baroreceptor actions and signals, induce ADH synthesis and release, as well as control salt and water appetite.

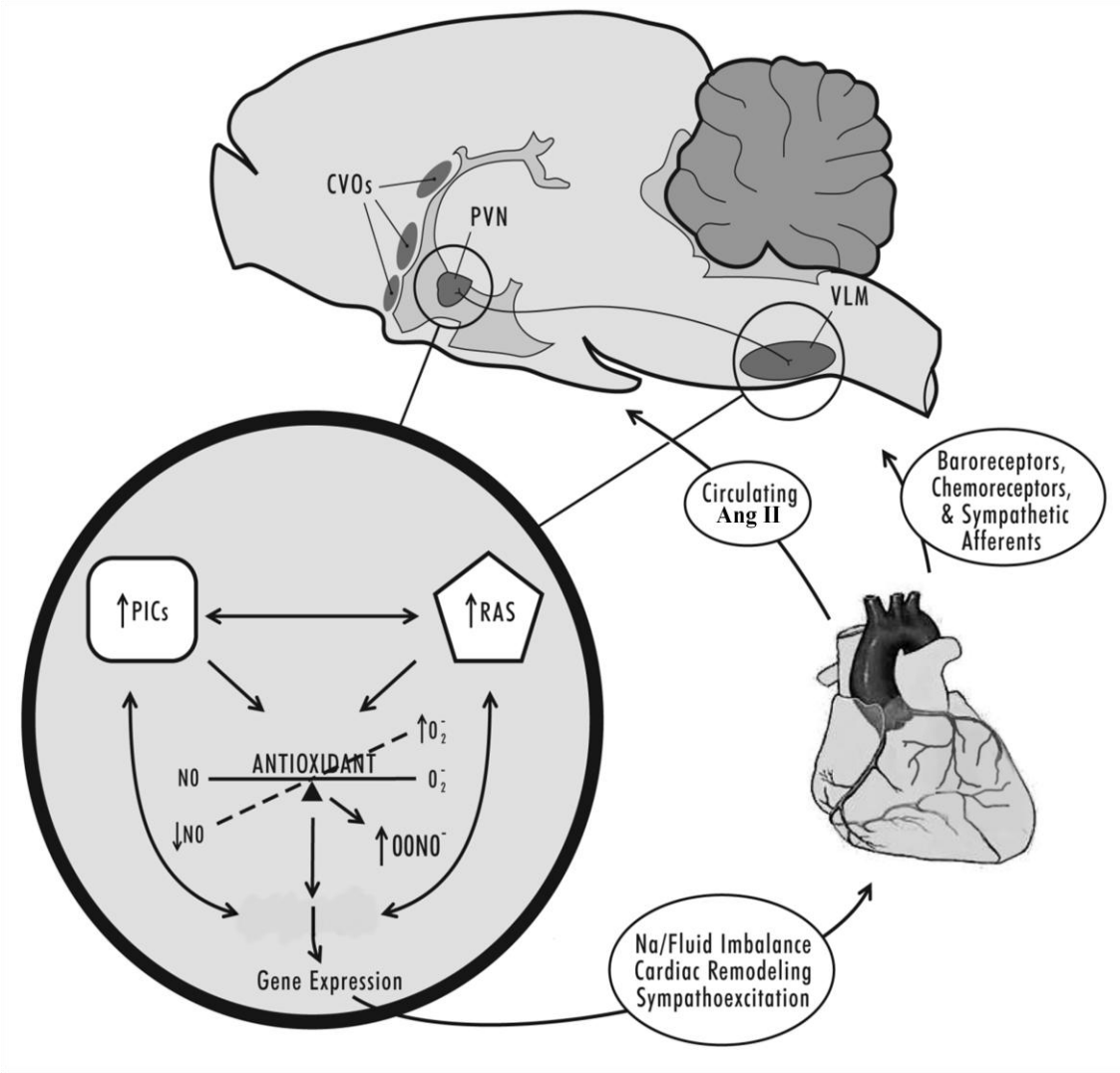

Figure 1.3. Schematic depicting the Ang II signaling pattern between the brain and the periphery. Increased Ang II signals to the brain via the blood brain barrier, whereupon it signals to various cardio-regulatory centers, such as the PVN. Local Ang II signaling subsequently causes an imbalance in PICs, ROS and the RAS thereby perpetuating the hypertensive state.

The proposed intracellular signaling mechanism whereby AT_1R activation causes a sympathetic response is unclear, but many studies have pointed to the role that PICs and ROS

play in this pathway (Peterson, Sharma et al. 2006; Harrison and Gongora 2009). For example, AT_1R activation activates membrane NADPH oxidases to produce ROS such as superoxide and hydrogen peroxide, and inhibits SODs. It is suspected that this NADPH oxidase activation goes through a phospholipase D/protein kinase C phosphorylating pathway (Seshiah, Weber et al. 2002). However, other investigations outline a p38 MAPK and ERK1/2 pathway, or through PIC activation of NFκB that increases ROS production (Chan, Hsu et al. 2005). Regardless, it is abundantly apparent that the combination of PIC, RAS and ROS cross-talk and responses within the brain drive the neurogenic portion of the hypertensive state.

Multiple regions within the brain have modulatory roles in the hypertensive response such as the VLM, subfornical organ (SFO), supraoptic nucleus (SON), nucleus tractus solitarius (NTS), area pastrema (AP) and organum vasculosum lamina terminalis (OVLT), but central within these signaling sites is the hypothalamic PVN (Dampney 1994; Coote 2005; Kang, Ma et al. 2009). Though much is known regarding autonomic regulation, the signaling pattern between these sites is complex and still not clearly understood (Coote 2005; Guyenet 2006). In brief, baroreceptor afferents and the vagus nerve signal the NTS of the medulla oblongata during pressure changes. NTS axons project to the magnocellular neurons of the PVN (involved in neuroendocrine control of ADH and fluid balance), while projections from the AP, OVLT and SFO (CVO regions that sense blood-borne neuropeptides such as Ang II and PICs) synapse on the parvocellular neurons of the PVN and the SON (Ericsson, Liu et al. 1995; Coote 2005). These parvocellular neurons project into the VLM where the signal travels down the medial column to regulate systemic sympathetic responses (Dampney 1994). These neuronal tracts denote the central role that the PVN has in sympathetic regulation, body fluid homeostasis via water and salt modulation, and the overall control of hypertensive responses. For instance,

studies microinjecting Ang II into the PVN of rats raise mean arterial blood pressure, which is attenuated following AT_1R antagonism (Zhu, Patel et al. 2002; Zhu, Gao et al. 2004). Also, chronic peripheral infusion of Ang II results in increased PIC, NFκB and ROS component expression in the PVN (Kang, Ma et al. 2009). The combination of these results points to a potential role that PICs, RAS and ROS in the PVN can play in contributing to the pathogenesis of hypertension. Better understanding these central mechanisms may also outline new pharmacological targets with which to dampen the debilitating effects of hypertension, making its study paramount.

STATEMENT OF THE PROBLEM AND SPECIFIC AIMS

The role of inflammation in hypertension is evident. Increased concentrations of PICs, ROS and RAS components are predominant during the pathogenesis of hypertension. Systemically, these factors contribute to cardiovascular changes, including cardiac hypertrophy, fibrosis and contractile dysfunction. Centrally, these same factors exacerbate those systemic changes through neuronal modulation of sympathetic activity, especially within the PVN, thereby altering pressure volume homeostasis during hypertension. Current therapeutic regimes typically target components of volume regulation, peripheral adrenergic receptor activity, or of the RAS. However, these options often fall short of controlling hypertension or the CVDs associated with hypertension. Therefore, a deeper understanding of altering mechanisms of hypertension is of pressing importance.

Recent evidence highlights the role that inflammation, specifically TNF, plays in hypertension and regulating the RAS, but the understanding as to how inflammation as a whole is regulated following Ang II or TNF activation, as well as the resultant consequences, is unclear. Based upon the literature and work from our lab, we hypothesized that inflammatory regulation

at the cytosolic and transcriptional level alters the systemic hypertensive response through regulation of the transcription factor NFκB and the dysregulation of components of the RAS. We tested this hypothesis *in vivo* through several sets of experiments. First, we needed to identfiy how TNF infusion alone would modulate the RAS in the heart and contribute to the hypertensive response. Second, we investigated the effect of chronic inhibition of histone modifications and the resultant effects on heart inflammation and NFκB, and its effect on the hypertensive response. Next, we hypothesized that central regulation of inflammation within the PVN, through TNF and NFκB, alters local PIC, RAS and ROS responses in propagating the systemic effects of the hypertensive state. Here we conducted a series of studies in which we centrally inhibited TNF or NFκB to determine the functional role of brain or PVN inflammation in the regulation of the RAS and in the perpetuation of the hypertensive response.

In order to explore these hypotheses, we preformed a series of *in vivo* experiments integrated with cardiovascular surgical, physiological and molecular techniques to elucidate our specific study aims:

Aim 1: Determine the role that systemic TNF infusion has on systemic cardiovascular, RAS and ROS responses in adult rat heart tissues and in blood pressure regulation.

Aim 2: Determine the role that histone deacetylases have on systemic cardiovascular NFκB, RAS and ROS responses in a chronically hypertensive rat model (spontaneously hypertensive rats; SHR).

Aim 3: Determine the role that central TNF inhibition would have on PVN PICs and RAS and ROS components in chronically Ang II-infused rats.

Aim 4: Determine the role that PVN specific NFκB has on regulating the RAS and ROS responses in the Ang II-induced hypertensive response.

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CHAPTER 2

TUMOR NECROSIS FACTOR – INDUCED OXIDATIVE STRESS REGULATES COMPONENTS OF THE RENIN-ANGIOTENSIN SYSTEM

INTRODUCTION

Cardiovascular diseases are commonly associated with increased expression of pro-inflammatory cytokines (PICs), including hypertension (Phillips and Kagiyama 2002; Ferrario and Strawn 2006). The renin-angiotensin system (RAS), the primary controlling mechanism in blood pressure regulation, also plays a major role in modulating the hypertensive response. Furthermore, studies from our lab and others have shown that in hypertension, PICs can lead to an increase in reactive oxygen species (ROS) and further increases in PIC, ROS and RAS transcription (Sriramula, Haque et al. 2008; Elks, Mariappan et al. 2009; Mariappan, Elks et al. 2009). Along with RAS components, PICs also activate hypertrophic mediators, which can result in cardiac hypertrophy and altered cardiac remodeling and function, potentially via a ROS-mediated mechanism (Tokuda, Kai et al. 2004; Kudo, Kai et al. 2009). Angiotensin II (Ang II), the effector peptide of the RAS, has been shown to incite the synthesis and release of PICs such as tumor necrosis factor-α (TNF), Interleukin (IL)-6 and chemokines (Funakoshi, Ichiki et al. 1999; Kalra, Sivasubramanian et al. 2002; Ruiz-Ortega, Ruperez et al. 2002), and both Ang II and PICs can increase ROS, which individually and combined can contribute to cardiac maladaptations, dysfunction and blood pressure response.

The actions of Ang II are typically understood through its classical pathway of angiotensin converting enzyme (ACE) and Ang II-Type 1 receptors (AT_1Rs), the pro-hypertensive/vasoconstrictive and deleterious ACE/Ang II/AT_1R pathway. However, a recent alternative axis of the RAS has been discovered that counteracts the actions of the pro-hypertensive RAS arm. This protective, anti-hypertensive/vasodilatory axis consists of ACE2, Ang (1-7) and the Mas receptor, amongst others (Donoghue, Hsieh et al. 2000; Tipnis, Hooper et al. 2000; Xu, Sriramula et al. 2011). Data suggests that the ACE2/Ang (1-7)/Mas arm may be

downregulated by an increase in Ang II (Xu, Sriramula et al. 2011), but the manner in which this dysregulation occurs, if through TNF, is unknown.

The PIC most central to the Ang II-driven hypertensive inflammatory responses is TNF, a multifarious cytokine that plays an important and diverse role in physiological and pathophysiological responses, including, but not limited to, inflammation, cell survival, growth, differentiation and apoptosis (Mann 2002). It is well established that there is an existent cross-talk in hypertension between the RAS, especially Ang II, and TNF (Brasier, Li et al. 1996; Sasamura, Nakazato et al. 1997; Kalra, Sivasubramanian et al. 2002; Arenas, Xu et al. 2004). We have recently demonstrated, along with others, the integral manner that Ang II-induced TNF functions to modulate blood pressure response and cardiac hypertrophy in Ang II-induced hypertension, but if and how TNF alone can modulate these RAS arms is uncertain (Muller, Shagdarsuren et al. 2002; Elmarakby, Quigley et al. 2006; Sriramula, Haque et al. 2008).

Early studies showed that the major source of vascular ROS is primarily NADPH oxidase derived superoxide (Griendling, Minieri et al. 1994; Mohazzab, Kaminski et al. 1994), which can be regulated by multiple mediators, including cytokines (Paravicini and Touyz 2008; Harrison and Gongora 2009). Moreover, an increased expression of NADPH oxidase was noted in cardiac tissue in Ang II-induced hypertensive animal models (Li, Gall et al. 2002; Paravicini and Touyz 2008). The NADPH oxidase subunit gp91*phox* (Nox2) is central to Ang II-induced cardiac hypertrophy (Bendall, Cave et al. 2002; Li, Gall et al. 2002). In Ang II-infused animal models and SHR rats, NADPH activity is increased and ROS generation is enhanced and these processes are mediated through AT_1Rs and are associated with the overexpression of vascular and cardiac NADPH oxidase subunits (Cifuentes, Rey et al. 2000; Heymes, Bendall et al. 2003; Kakishita, Nakamura et al. 2003). These studies support a role for NADPH oxidase-derived superoxide and

increased oxidative stress in the pathogenesis of Ang II-induced hypertension. Within the heart, ROS signaling elicits numerous responses, including cardiac hypertrophy and fibrosis, which can be triggered by TNF and can be inhibited through the use of anti-oxidants (Seddon, Looi et al. 2007; Paravicini and Touyz 2008). Furthermore, sub-pressor doses of Ang II can also induce cardiac hypertrophy and interstitial fibrosis in mice, which can also be blunted in hearts lacking gp91*phox* (Bendall, Cave et al. 2002). Interstitial cardiac fibrosis and remodeling was also inhibited in Nox2 knockout mice subjected to aortic banding, Ang II or aldosterone infusion, or in animals treated with apocynin or diphenylene iodinium (NADPH oxidase inhibitors) (Bendall, Cave et al. 2002; Park, Park et al. 2004; Touyz, Mercure et al. 2005; Grieve, Byrne et al. 2006; Johar, Cave et al. 2006; Paravicini and Touyz 2008).

The signaling pattern from Ang II to TNF is evident, but how TNF alone can act, if at all, on components of the RAS and on blood pressure response is uncertain. With these considerations, we hypothesized that daily TNF injections would contribute to cardiac tissue RAS modulation and blood pressure response, possibly through a ROS mechanism. In order to conduct this study, we injected TNF for 5 consecutive days, during which time we recorded blood pressure via radio-telemetry probes and conducted echocardiographic assessment of cardiac function. At the conclusion of the study, left ventricle (LV) tissue was collected for assessment of RAS and ROS component expression. For determination of the role of ROS as a signaling mediator between TNF and RAS component expression, along with TNF treatment, we simultaneously administered either Tempol, a superoxide dismutase (SOD) mimetic, or etanercept, a soluble TNF receptor mimetic. While neither TNF nor the treatments had any effect on blood pressure regulation, alterations were observed in cardiac function and RAS component expression, ultimately demonstrating that the TNF can increase cardiac dysfunction (via its

function as a negative inotrope) and pro-hypertensive/vasoconstrictive RAS components, and decrease anti-hypertensive RAS component expression, which was reversed following Tempol or etanercept treatment. This study also demonstrates that ROS are a signaling mediator between TNF and the RAS and cardiac changes. More importantly, it shows that TNF can differentially regulate the damaging and protective arms of the RAS, potentially via a ROS mechanism, which may be a tipping point in the hypertensive response.

MATERIALS AND METHODS

Animals. Adult male Sprague-Dawley rats (250-350 grams) were used in this study. Animals were housed in a temperature-controlled room (25 ± 1^{o}C) and maintained on a 12:12 hour light:dark cycle with free access to water and food. All animal and experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Louisiana State University in compliance with NIH guidelines.

Experimental Protocol. The rats were implanted with radio-telemetry transmitters to measure blood pressure, and injected daily with TNF (40µg/kg, i.p.) dissolved in saline or saline alone for 5 consecutive days. This TNF dose was based on unpublished dose-response studies as well as previous publications from our laboratory and others (Michie, Sherman et al. 1989; Bozkurt, Kribbs et al. 1998; Mariappan, Soorappan et al. 2007; Mariappan, Elks et al. 2009). Simultaneously, a subset of TNF and saline-treated rats received either Tempol (Temp; 300µmol/kg, p.o.), a SOD mimetic, or Etanercept (ETN; 1mg/kg/day, s.c.), a soluble TNF receptor mimetic. Saline-treated rats receiving Temp or ETN were unchanged from saline-treated controls and as such were combined together for the final analysis. The study consisted of four experimental groups: 1) Controls (saline alone, saline+Tempol and saline+etanercept); 2) TNF; 3) TNF+Temp; and 4) TNF+ETN. Echocardiographic assessment was carried out at baseline and

at the conclusion of the study. After five days, the rats were euthanized and the hearts were removed for further analysis.

Blood Pressure Measurement. Blood pressure was measured continuously in conscious rats implanted with radio-telemetry transmitters (Model TA11PA-C40, Data Sciences International, St. Paul, MN). Rats were anesthetized with a ketamine (90 mg/kg) and xylazine (10 mg/kg) mixture (i.p.) and placed dorsally on a heated surgical table. An incision was made on the ventral surface of the left leg, the femoral artery and vein were exposed and bluntly dissected apart. The femoral artery was ligated distally, and another was used to temporarily interrupt the blood flow. The catheter tip was introduced through a small hole in the femoral artery, advanced ~6 cm into the abdominal aorta such that the tip was distal to the origin of the renal arteries, and secured into place. The probe body was placed into the abdominal cavity and secured to the abdominal wall. The abdominal musculature was sutured and the skin layer closed following implantation. Rats received benzathine penicillin (30000 U, i.m.) and buprenorphine (0.1 mg/kg, s.c.) immediately following surgery and 12hr postoperatively and allowed to recover for seven days.

Echocardiographic Assessment of LV Hypertrophy. Echocardiography was obtained at baseline, and repeated at the end of the treatment period. Echocardiogram was performed as described previously (Mariappan, Elks et al. 2009). Briefly, transthoracic echocardiography was performed under isoflurane anesthesia, using a Toshiba Aplio SSH770 (Toshiba Medical, Tustin, California) fitted with a PST 65A sector scanner (8 MHz probe) which generates two-dimensional images at a frame rate ranging from 300-500 frames per second. Left ventricle wall and chamber parameters, fractional shortening (%FS) and Tei index were measured and calculated to assess cardiac hypertrophy and cardiac function.

Detection of Total ROS and Superoxide ($O_2^{\bullet-}$) in LV Heart Tissue. One of the most sensitive and definitive methods of superoxide production is electron spin resonance (EPR). In this study, we utilized an established technique for total ROS detection in tissue using EPR and spin traps (Mariappan, Elks et al. 2009). Different spin probes were used for the EPR studies. 1-hydroxy-3-methoxycarbonyl-2, 2, 5, 5-tetramethylpyrrolidine (CMH) was used to measure the $O_2^{\bullet-}$ levels. All ESR measurements were performed using an EMX EPR eScan BenchTop spectrometer and super-high quality factor (Q) microwave cavity (Bruker Company, Germany). **Sample Preparation for EPR Studies:** The dissected LV tissue from each animal was placed into a 24-well plate containing Kreb's HEPES buffer (KHB) (20mM, pH 7.4). Tissue pieces were then washed twice with the same buffer to remove any trace contamination. Samples were then incubated at 37^{o}C with specific spin probes for 30 minutes. **Total tissue ROS production:** Total ROS was determined as previously described (Mariappan, Elks et al. 2009). Tissue pieces were incubated at 37^{o}C with CMH (200 μM) for 30 minutes. Aliquots of the incubated probe media were then taken in 50 μl glass capillary tubes (Noxygen Science Transfer and Diagnostics, Elzach, Germany) for determination of total ROS production, under the following EPR settings: field sweep 50 G; microwave frequency 9.78 GHz; microwave power 20 mW; modulation amplitude 2 G; conversion time 327 ms; time constant 655 ms; receiver gain 1 x 10^5. For superoxide production, samples were pre-incubated at 37^{o}C with PEG-SOD (50 U/ml) for 30 minutes, then CMH (200uM) for an additional 30 minutes. Aliquots of the incubated probe media were taken in 50 μl glass capillary tubes for determination of total superoxide production. Addition of PEG-SOD to CMH allowed competitive inhibition of the $O_2^{\bullet-}$-CMH oxidation reaction by the quenching of $O_2^{\bullet-}$ radicals. Since it is cell permeable, PEG-SOD can competitively inhibit the CMH-$O_2^{\bullet-}$ interaction both intra-cellularly and extra-cellularly, thus

allowing accurate measurement of total tissue $O_2^{\bullet-}$ production. To determine actual total tissue superoxide production, the values obtained from incubation with PEG-SOD and CMH were subtracted from the values obtained from incubation with CMH only.

RNA Isolation and Real-Time RT-PCR. Total RNA was extracted from the LV using TRI reagent (Invitrogen), and cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad) as previously described (Guggilam, Haque et al. 2007; Sriramula, Haque et al. 2008). The mRNA expression levels of gp91*phox*, angiotensinogen, renin, angiotensin converting enzyme (ACE), ACE2, AT_1R and the Mas receptor were determined using previously published specific custom made primers (Guggilam, Haque et al. 2007; Sriramula, Haque et al. 2008; Agarwal, Haque et al. 2009; Elks, Mariappan et al. 2009; Mariappan, Elks et al. 2009). GAPDH was used as the housekeeping gene. Real-time RT-PCR (qRT-PCR) was performed in 384 well PCR plates using Bio-Rad PCR Master Mix (The iTaq SYBR™ Green Supermix with ROX) and the ABI Prism 7900 sequence detection system (Applied Biosystems). The PCR cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles (15 s at 95°C, 1 min, at 60°C). A dissociation step (15 s at 95°C, 15 s, at 60°C and 15 s at 95°C) was added to check the melting temperature of the specific PCR product.

Western Blot. Protein was extracted from LV samples in ice-cold buffer (10mM Tris-HCl, pH 7.4, 1mM EDTA, 1% Nonidet P-40, 0.1% sodium deoxycholate, and 0.1% SDS) containing a protease inhibitor cocktail (Roche). The protein content in the supernatant was determined using a detergent-compatible protein assay (Bio-Rad). Protein samples (25μg) were resolved in 10% SDS-polyacrylamide gel. The protein expression of gp91*phox* and AT_1R in the heart was analyzed by western blot as previously described (Sriramula, Haque et al. 2008) with the use of anti-gp91*phox*, anti-AT_1R (Abcam) and the Mas receptor (Santa Cruz) antibody (1:200 dilution).

Bands were normalized to α-Actinin or GAPDH (Santa Cruz). Bands were analyzed for graphical and statistical densitometric representation using ImageJ.

Measurement of Plasma TNF. At the end of the study, blood was collected in chilled EDTA tubes, plasma was separated and stored at -80°C until assayed. Circulating levels of TNF were quantified in the plasma using a commercially available rat TNF ELISA kit (Biosource, Camarillo, CA) according to manufacturer's instructions.

Measurement of Plasma Catecholamines. At the end of the study, blood was collected in chilled EDTA tubes, plasma was separated and stored at -80°C until assayed. Circulating levels of norepinephrine (NE) and epinephrine (EPI) were measured using high performance liquid chromatography (HPLC). Plasma samples were prepared by adding activated alumina, Tris buffer, EDTA and internal standard DHBA, along with 0.5 ml of rat plasma. The samples were centrifuged and the supernatant was separated and rinsed 2x in ultra pure water and filtered through a Millipore filter (Ultrafree MC UFC30GV00, Millipore Corp.). Prepared samples were then filtered and injected into an Eicom HTEC-500 system fitted with an HPLC-ECD, as previously described (Guggilam, Haque et al. 2007).

Localization of Ang II by Immunofluorescence. Heart tissues were prepared as previously described (Guggilam, Haque et al. 2007). The sections were treated with primary antibody against Ang II (1:500 dilution, anti-goat) (Santa Cruz). Negative control sections were incubated with secondary antibody alone.

Statistical Analysis of Data. All results are expressed as mean±SEM. For statistical analysis of the data, student's *t* test, one-way ANOVA or repeated measures ANOVA followed by Bonferroni's *post hoc* test was performed using GraphPad Prism version 5.0 for Windows

(GraphPad Software, San Diego California, USA) to determine differences among groups. A value of $p<0.05$ was considered statistically significant.

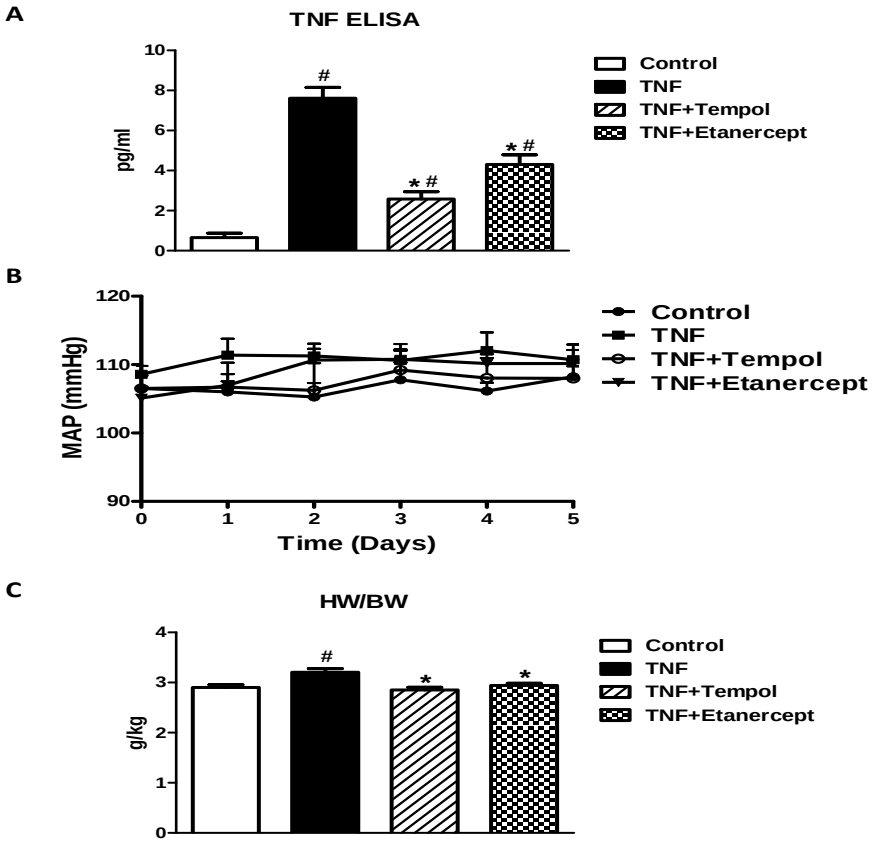

Figure 2.1 Effects of TNF on circulating TNF, mean arterial blood pressure (MAP) and heart weight/body weight (HW/BW). Plasma TNF concentration were elevated after 5 consecutive days of TNF injection versus controls and co-administration of Tempol (TNF+Temp) or etanercept (TNF+ETN) (**A**). MAP was unaffected by TNF alone or in TNF+Temp or TNF+ETN groups throughout the study period (**B**). HW/BW ratio as assessed upon sacrifice shows an increase in cardiac mass after 5-day TNF treatments versus controls (**C**). Tempol and etanercept reversed this increase in cardiac mass. n=9-10 per group, #$p<0.05$ vs Control, *$p<0.05$ vs TNF.

RESULTS

Daily TNF Injections have no Effect on Blood Pressure but alters Cardiac Function in Treated Rats. The effect of daily TNF injections was examined at the conclusion of the 5-day

study period to determine TNF plasma concentrations (Figure 2.1A). Using a TNF ELISA kit, plasma concentrations were, as expected, much higher in TNF-treated as compared to controls (7.61±0.53 v 0.65±0.22 pg/ml, respectively; $p<0.05$). These circulating levels of TNF were reduced following Tempol (2.58±0.36 pg/ml; $p<0.05$) or etanercept (4.3±0.48 pg/ml; $p<0.05$) co-administration with TNF. This demonstrates that the TNF injections induced increases in endogenous TNF. The endogenously produced TNF was also reduced following ROS/superoxide reduction, thereby linking the TNF-induced TNF signal propagation to ROS intermediaries.

Table 2.1. Effects of TNF on cardiac function as assessed by echocardiography.

Table 2.1	**Control**	**TNF**	**TNF+Temp**	**TNF+ETN**
n	9	10	10	10
IVSTd (mm)	1.36±0.02	1.59±0.05#	1.37±0.05*	1.41±0.03*
IVSTs (mm)	2.26±0.03	2.48±0.12	2.50±0.06	2.25±0.03
LVIDd (mm)	6.57±0.08	7.10±0.21	7.31±0.13	7.28±0.13
LVIDs (mm)	5.27±0.10	5.69±0.18#	5.06±0.13*	4.78±0.15*
LVPWTd (mm)	1.37±0.11	1.57±0.07	1.38±0.03	1.38±0.03
LVPWTs (mm)	2.25±0.13	2.15±0.06	2.25±0.16	2.15±0.11
%FS	55.2±1.92	39.7±0.95#	50.2±1.12*	43.8±0.77
Tei	0.29±0.03	0.38±0.11#	0.30±0.02*	0.32±0.01
HR	327±7.46	366±10.1#	329±3.78*	337±4.68*

IVSTd/s, interventricular septal thickness at end-diastole and end-systole, respectively; LVIDd/s, left ventricular internal diameter at end-diastole and end-systole, respectively; LVPWTd/s, left ventricular posterior wall thickness at end-diastole and end-systole, respectively; %FS, fractional shortening; HR, heart rate; n=9-10 in each group, #$p<0.05$ vs Control, *$p<0.05$ vs TNF.

To assess the effect of 5-day TNF injections on mean arterial pressure (MAP), blood pressure was measured using a radio-telemetry system. There was no change in MAP from baseline over the 5-day TNF injection period (Figure 2.1B). Moreover, TNF+Temp and TNF+ETN had no effect on MAP versus the saline-treated controls, with all rat groups maintaining a MAP between 105-112 mmHg during the course of the study. Interestingly,

though there were no appreciable differences in the MAP, the heart weight/body weight (HW/BW) ratio was increased in TNF-treated rats versus controls (3.2±0.07 v 2.9±0.05 g/kg, respectively; $p<0.05$), which was reversed following Tempol (2.85±0.05 g/kg; $p<0.05$) and Etanercept (2.94±0.04 g/kg; $p<0.05$) treatments (Figure 2.1C). Furthermore, interventricular septal thickness at end-diastole (IVSTd) was significantly increased, while IVST in end-systole (IVSTs) followed a similar trend (Table 2.1), confirming the HW/BW data and indicating TNF-induced cardiac hypertrophy. Also noted was that TNF treatments, possibly via its function as a negative inotrope, reduced %FS and raised HR and the Tei index, signaling cardiac dysfunction.

TNF Increases Total ROS and gp91*phox* in the LV of TNF-Treated Rats. As TNF is often associated with increases in ROS, both total ROS and superoxide as assessed by EPR (Figure 2.2) and the expression of gp91*phox* (Figure 2.3) were examined in the LV. TNF-treated rats experienced a significant increase in total ROS and superoxide when compared to saline-infused control rats (total ROS: 0.35±0.006 v 0.11±0.006 mM/mg protein/min, respectively; $p<0.05$; and superoxide: 0.122±0.007 v 0.021±0.006 mM/mg protein/min, respectively; $p<0.05$). TNF+Temp and TNF+ETN normalized these ROS

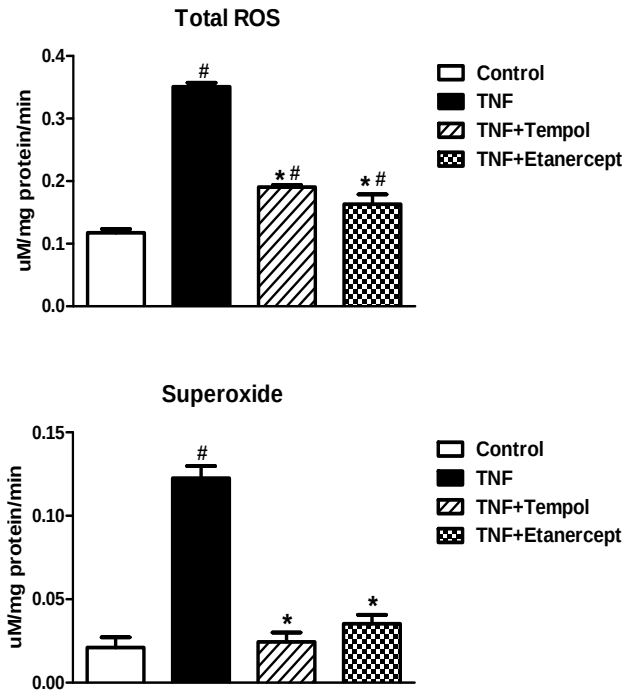

Figure 2.2. Effect of TNF on ROS. Total ROS and superoxide as measured by electron paramagnetic spin resonance (EPR) were increased in 5-day TNF-treated rats. This was reduced in TNF+Temp and TNF+ETN groups after 5 days. n=6 in each group, #$p<0.05$ vs Control, *$p<0.05$ vs TNF.

increases (total ROS reduced to: 0.19±0.003 and 0.16±0.015 mM/mg protein/min, respectively; $p<0.05$; and superoxide reduced to: 0.024±0.005 and 0.035±0.005 mM/mg protein/min, respectively; $p<0.05$). Furthermore, the mRNA expression of gp91*phox*, the major catalytic subunit of NADPH oxidase and a major ROS contributor, was increased in TNF-treated rats versus the controls (Figure 2.3A). This was reduced in TNF+Temp and TNF+ETN rats. The reduction in protein expression was further confirmed by western blot of the LV tissue (Figure 2.3B). These results indicate that treatment with Tempol and Etanercept had beneficial effects in reducing TNF-induced oxidative stress in the LV heart tissue in TNF-infused rats.

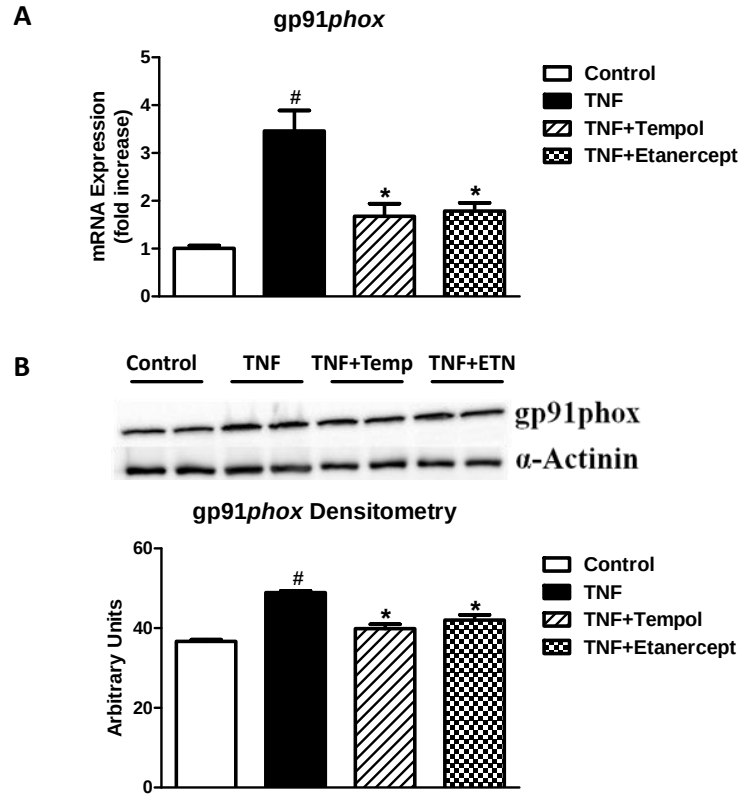

Figure 2.3. Effects of TNF on gp91*phox*. The catalytic subunit of NADPH oxidase, gp91*phox*, was assessed via RT-PCR (**A**) and western blot (**B**). Both mRNA and protein showed a similar pattern of increase in TNF-treated rats versus controls. The TNF-induced changes were reduced following Tempol or etanercept co-administration. n=6-8 in each group, #$p<0.05$ vs Control, *$p<0.05$ vs TNF.

Components of the RAS Are Altered in the LV Following TNF Injections. In an attempt to understand the link between the RAS and inflammation, several components of the RAS were measured. The mRNA expression of the pro-hypertensive/vasoconstrictive RAS components angiotensinogen, renin, ACE and AT_1R (Figure 2.4) are increased following 5 days of TNF treatment when compared to controls, while the anti-hypertensive/vasodilatory components ACE2 and the Mas receptor were decreased (Figure 2.6A). These trends were reversed following treatment with Tempol or etanercept. Moreover, the protein levels of AT_1R and the Mas receptor as measured via western blot followed similar patterns to their respective mRNA expressions, (Figure 2.5A, 2.6B). The levels of Ang II were further assessed within the LV tissues using immunofluorescence. Ang II was similarly increased in TNF-treated rats, but decreased in TNF+Temp and TNF+ETN rats (Figure 2.5B). This indicates that TNF differentially alters injurious and protective RAS component expression within cardiac tissue, which can be reversed following TNF or superoxide inhibition.

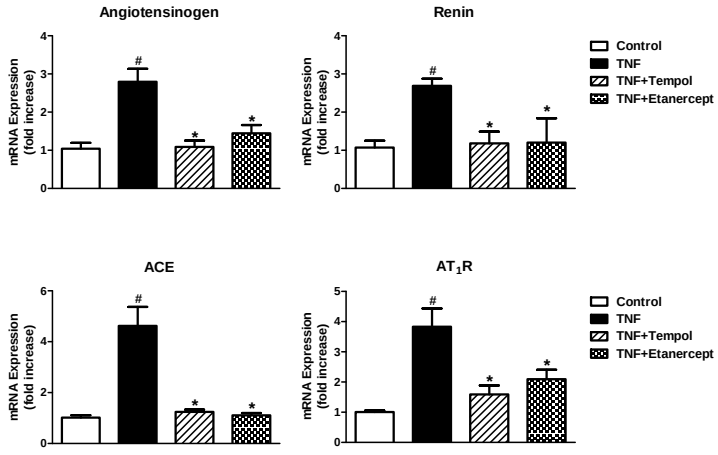

Figure 2.4. Effects of TNF administration on pro-hypertensive components of the RAS system. mRNA expression for angiotensinogen, renin, angiotensin converting enzyme (ACE) and AT_1R all followed similar patterns in the left ventricular tissues of treated rats. After 5 days, TNF injections increased these pro-hypertensive components of the RAS versus controls. These increases were normalized in TNF+Temp and TNF+ETN groups. n=6-8 in each group, #p<0.05 vs Control, *p<0.05 vs TNF.

Circulating Catecholamines Increase in TNF-Infused Rats. To assess the effect of daily TNF injections on catecholamine release, circulating plasma NE and EPI were determined by HPLC (Figure 2.7). TNF injections increased NE and EPI versus the control rats (1341±53.9 v 704.1±85.3 pg/ml and 459±40.7 v 185.7±12.4 pg/ml, respectively; $p<0.05$). These elevated levels were decreased in TNF+Temp and TNF+ETN groups, signifying that TNF plays a role, possibly through a RAS and ROS-mediated mechanisms, in elevating circulating catecholamines and contributing to increased sympathetic activity, cardiac dysfunction and remodeling.

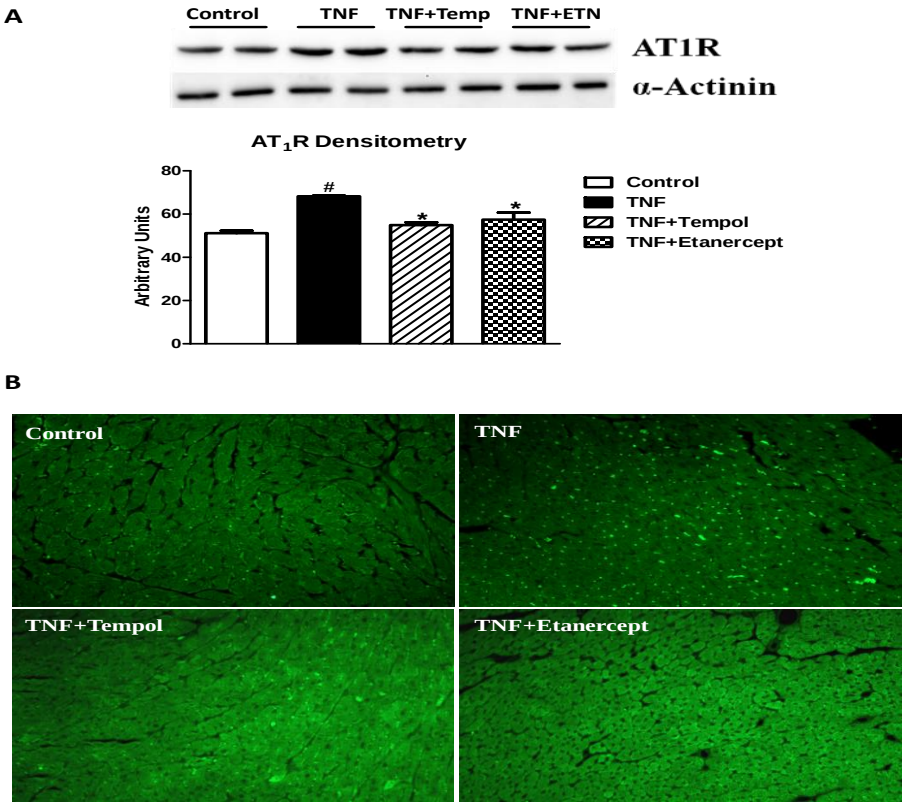

Figure 2.5. Effects of TNF on AT_1R protein expression and Ang II in left ventricular tissues. Western blot and densitometric analysis reveals that similar to the mRNA expression of AT_1R, TNF increases its abundance when compared to controls (**A**). Likewise, Tempol or etanercept attenuated these changes. Immunofluorescence (Original magnification x20) staining of Ang II in cardiac tissue sections shows an increase in Ang II in the TNF-treated rats (**B**). Co-administration with Tempol or etanercept eliminated this increase in Ang II within the heart. n=6-8 in each group for AT_1R protein, n=3-4 for immunofluorescence images, #$p<0.05$ vs Control, *$p<0.05$ vs TNF.

DISCUSSION

The major findings in this study are as follows: 1) TNF injections for 5 days had no effect on mean arterial blood pressure, but TNF did increase cardiac hypertrophy and negatively altered cardiac function as seen with a reduction in fractional shortening and an increase in the Tei index; 2) TNF injections increased total ROS and superoxide in the LV of treated rats; 3) TNF-infused rats showed an elevation in pro-hypertensive/ vasoconstrictive RAS component expression and a decrease in anti-hypertensive/vasodilatory RAS component expression, as well as an increase in the circulating catecholamines NE and EPI; and 4) TNF-infused rats treated with Tempol or Etanercept prevented these changes observed following TNF injection alone. These findings suggest that TNF can act on the opposing components of the RAS within the LV, possibly through a ROS-driven

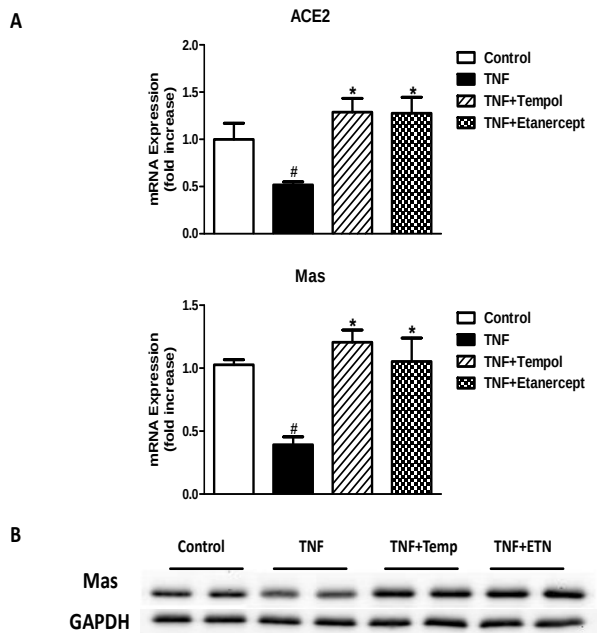

Figure 2.6. Effects of TNF on ACE2 and Mas receptor expression in left ventricular tissues. The mRNA expression of ACE2 and Mas decreases following TNF injection when compared to controls (**A**). Tempol or etanercept reversed these changes and returned them to normal levels. Western blot against the Mas receptor in cardiac tissues shows a decrease in the protein levels in TNF-treated rats (**B**). Co-administration with Tempol or etanercept reversed this decrease within the heart. n=6-8 in each group for AT_1R protein, n=3-4 for immunofluorescence images, #p<0.05 vs Control, *p<0.05 vs TNF.

mechanism, and negatively affect cardiac function and cardiac mass while not effecting pressure responses in treated rats.

Hypertension is increasingly accepted as involving an inflammatory component (Ferrario and Strawn 2006; Sriramula, Haque et al. 2008; Cardinale, Sriramula et al. 2010). Furthermore, the RAS plays a key role in the development and maintenance of hypertension (Brunner 2001; Ruiz-Ortega, Lorenzo et al. 2001), and Ang II, the effector peptide of the RAS, has been shown to stimulate the production and release of PICs such as TNF, IL-6 and chemokines (Funakoshi, Ichiki et al. 1999; Kalra, Sivasubramanian et al. 2002; Ruiz-Ortega, Ruperez et al. 2002). Components of the ACE2/Ang (1-7)/Mas RAS axis are also downregulated during hypertensive disease progression (Xu, Sriramula et al. 2011). Recently, work from our lab demonstrated the involvement of Ang II-induced TNF in the Ang II-mediated effects on the hypertensive response (Sriramula, Haque et al. 2008). Changes observed included an increase in salt appetite and water retention, therefore unbalancing systemic volume homeostasis, as well as increased cardiac hypertrophy and the overall perpetuation of the hypertensive state. Others have also observed the existence of a potential cross-talk between the RAS and TNF. *In vitro* studies showed the production of TNF following cardiomyocyte and

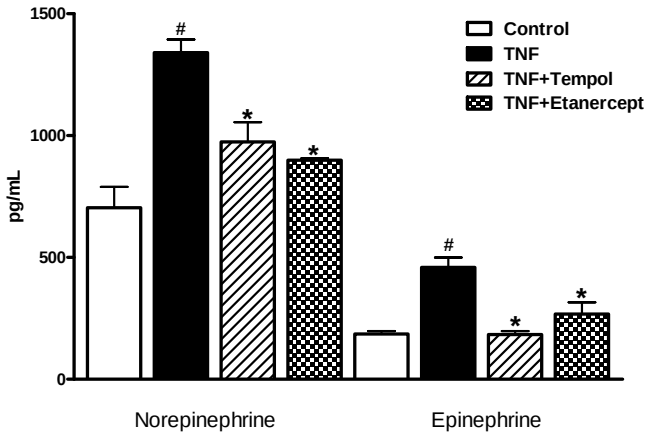

Figure 2.7. Effects of TNF on circulating catecholamines. The circulating plasma norepinephrine (NE) and epinephrine (EPI) levels were determined by HPLC. TNF injection increased NE and EPI versus controls, which was decreased in TNF+Temp and TNF+ETN groups. n=6-8 in each group, #$p<0.05$ vs Control, *$p<0.05$ vs TNF.

fibroblast cell culture treatments with Ang II, and that neonatal rat cardiac fibroblast culture treatments with TNF caused an increase in AT_1R expression (Gurantz, Cowling et al. 1999; Yokoyama, Sekiguchi et al. 1999; Kalra, Sivasubramanian et al. 2002). *In vivo* studies have also linked PICs and the RAS, including demonstrating that chronic AT_1R inhibition in hypertensive and heart failure patients decreased circulating TNF and that TNF blockade prevented renal damage and lowered blood pressure in a genetic model of hypertensive rats (Cottone, Vadala et al. 1998; Tsutamoto, Wada et al. 2000; Muller, Shagdarsuren et al. 2002; Elmarakby, Quigley et al. 2006). Combined, these changes suggest an intimate connection between the RAS and PICs, especially TNF, in the hypertensive response, in that Ang II activates TNF, which is speculated to further increase pro-hypertensive RAS expression. In this study, we looked at the possible reverse signaling pattern of the afore mentioned mechanism. Though not altering blood pressure, we show that five days of continuous TNF injections can differentially regulating RAS components such as Ang II, ACE2, AT_1 and the Mas receptors by decreasing the vasodilatory components and increasing the vasoconstrictive components of the RAS. In time, these RAS component dysregulations, especially the increase in Ang II, could lead to arterial pressure increases, but the current study was not carried out to that point.

The role of ROS in the pathogenesis of hypertension is well established in many experimental hypertensive models (Harrison and Gongora 2009). Activation of NADPH oxidase is regulated by many vasoactive hormones, cytokines, growth factors (platelet-derived growth factor, transforming growth factors, etc.) and mechanical stimuli such as shear stress and stretch (Paravicini and Touyz 2008; Harrison and Gongora 2009). Compared to their normotensive controls, SHR and DOCA salt-sensitive rats show an increase in the production of superoxide in cardiac, renal and vascular tissues, primarily through NADPH oxidase activity (Zalba, Beaumont

et al. 2000; Zhang, Griendling et al. 2005). As seen in the current study, increased ROS production, with an increased expression of NADPH oxidase, was noted in cardiac tissue in both a pressure overload model of cardiac hypertrophy and in Ang II hypertensive animal models (Li, Gall et al. 2002; Paravicini and Touyz 2008). The role of the gp91*phox* subunit (Nox2) of NADPH oxidase in Ang II-induced cardiac hypertrophy is pivotal. Studies conducted on mice lacking gp91*phox* showed that myocardial NADPH oxidase and superoxide levels were not as elevated in these animals as compared to their wild-type controls (Bendall, Cave et al. 2002), supporting the fact that NADPH oxidase is the major source of ROS in cardiomyocytes in hypertrophy due to a chronically increased blood pressure, which can progress to heart failure (Li, Gall et al. 2002). In Ang II-infused animal models and SHR rats, NADPH activity is increased and ROS generation is enhanced; these processes are mediated through AT_1Rs and associated with overexpression of vascular and cardiac NADPH oxidase subunits (Cifuentes, Rey et al. 2000; Heymes, Bendall et al. 2003; Kakishita, Nakamura et al. 2003). An *in vitro* study using cardiomyocyte cell cultures, Ang II stimulation increases both mRNA and protein expression of gp91*phox* (Griendling, Sorescu et al. 2000). These studies, combined with the current data depicting an elevation in gp91*phox* expression and total ROS and superoxide, supports a role for NADPH oxidase-derived ROS and increased oxidative stress in the pathogenesis of Ang II-induced hypertension through a TNF-driven mechanism, but the role of ROS on the protective ACE2/Ang (1-7)/Mas RAS axis is vague.

Within the heart, ROS signaling elicits numerous responses, including cardiac hypertrophy (Seddon, Looi et al. 2007; Paravicini and Touyz 2008), though its effects by and through the separate arms of the RAS are only now coming under scrutiny. Oxidative stress is also a well known profibrotic mediator in many tissue systems, including the heart. Moreover,

excessive interstitial fibrosis is an injurious aspect of cardiac hypertrophy. These changes, in turn, cause the slow progression of cardiac maladaptive remodeling and dysfunction, as observed here following TNF injection and a subsequent decrease in fractional shortening and an increase in the Tei index. In cardiomyocyte cultures, Ang II, endothelin 1, NE, TNF and mechanical stretch can all induce cellular hypertrophy, including an increase in ROS production (Cave, Grieve et al. 2005), which can be inhibited through the use of antioxidants. Ang II-treated primary rat cardiomyocytes also demonstrated increased Rac1 (a small signaling G protein) activation, superoxide production, and cell hypertrophy. These changes were blocked by adenoviral introduction of dominant negative Rac1, CuZn-SOD and by small interfering RNA (siRNA) directed to the gp91*phox* subunit of NADPH oxidase, suggesting that Ang II-activation of gp91*phox* contributes to cardiac hypertrophy (Hingtgen, Tian et al. 2006). However, sub-pressor doses of Ang II also induce cardiac hypertrophy and interstitial fibrosis in mice, which can also be blunted in hearts lacking gp91*phox* (Bendall, Cave et al. 2002). Interstitial cardiac fibrosis and remodeling was also inhibited in Nox2 knockout mice subjected to aortic banding, Ang II or aldosterone infusion, or in animals treated with apocynin or diphenylene iodinium (NADPH oxidase inhibitors) (Bendall, Cave et al. 2002; Park, Park et al. 2004; Touyz, Mercure et al. 2005; Grieve, Byrne et al. 2006; Johar, Cave et al. 2006; Paravicini and Touyz 2008). Similarly, TNF alone caused cardiac alterations such as cardiac hypertrophy, decreased fractional shortening and an increased Tei index, but following treatment with Tempol and etanercept, these changes were reversed. Furthermore, NE and EPI, molecules that can participate in altered cardiac function and remodeling (Badenhorst, Veliotes et al. 2003; Cave, Grieve et al. 2005), as well as in the generalized sympathetic drive associated with hypertension, was increased in TNF-infused rats, but decreased following Tempol or etanercept treatment. This

data reinforces the role that ROS plays in the TNF-induced signaling pattern for increased cardiac dysfunction and maladaptive alterations, possibly through the dysregulation of RAS component expression.

It is well understood that the RAS and Ang II can increase the production of PICs and contribute to the hypertensive state, and that blocking the PIC TNF can prevent some of the deleterious changes associated with hypertension and the ACE/Ang II/AT_1R RAS axis (Funakoshi, Ichiki et al. 1999; Kalra, Sivasubramanian et al. 2002; Muller, Shagdarsuren et al. 2002; Ruiz-Ortega, Ruperez et al. 2002; Elmarakby, Quigley et al. 2006). The suggested mechanism potentially involves a positive feed-forward system whereby Ang II increases TNF and ROS, which in turn further increases injurious ACE/Ang II/AT_1R RAS axis components, and decreases protective ACE2/Ang (1-7)/Mas RAS components. Therefore, we aimed to determine if administration of TNF alone could regulate components of the RAS and if so, was this mechanism achieved through modulation of ROS. As detailed above, TNF injection increased components of the deleterious axis of the RAS including angiotensinogen, ACE, Ang II and the AT_1R. TNF injection also increased gp91*phox*, total ROS and superoxide, and decreased the protective RAS components ACE2 and the Mas receptor. These changes were reversed following administration of the TNF receptor mimetic etanercept, indicating that the TNF-driven pathway required the additional production of endogenous TNF, and the use of the SOD mimetic Tempol demonstrates that the TNF-induced TNF-driven pathway also requires ROS, especially superoxide, to contribute to the dysregulation of the individual RAS arms and the propagation of mediators that can contribute to the hypertensive state. In conjunction with the understanding that Ang II can cause an increase in TNF and the studies detailing that use of PIC and ROS inhibitors can reduce the hypertensive response, we demonstrate here that the ROS

involved appears to evolve through a TNF mechanism, therefore highlighting the Ang II/TNF/ROS pathway rather than an Ang II/ROS/TNF pathway, and that this in turn reduces the protective RAS axis (ACE2/Ang (1-7)/Mas) while upregulating the more injurious aspects of the pro-hypertensive/vasorestrictive RAS arm (ACE/Ang II/AT_1R). This study underscores the role that TNF plays in the ROS-induced dysregulation of the RAS and the ROS driven mechanism behind TNF-induced cardiac dysfunction, spotlighting the role that PICs, such as TNF, may play in the differential RAS regulation in the pathogenesis of the hypertensive response.

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CHAPTER 3

HDAC INHIBITION ATTENUATES INFLAMMATORY, HYPERTROPHIC AND HYPERTENSIVE RESPONSES IN SPONTANEOUSLY HYPERTENSIVE RATS*

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INTRODUCTION

Essential hypertension is a condition associated with increased expression of pro-inflammatory cytokines (PICs) (Phillips and Kagiyama 2002; Ferrario and Strawn 2006). Studies from our lab and others have shown that PICs lead to an increase in reactive oxygen species (ROS), which up-regulates Nuclear Factor-*kappa*B (NFκB) activity, thus further increasing PIC and ROS transcription and amplifying their subsequent actions (Sriramula, Haque et al. 2008; Elks, Mariappan et al. 2009; Mariappan, Elks et al. 2009). Along with renin-angiotensin system (RAS) components, PICs also activate hypertrophic mediators, which can result in cardiac hypertrophy and altered cardiac remodeling and function (Tokuda, Kai et al. 2004; Kudo, Kai et al. 2009).

There are many triggers of hypertensive-induced inflammation resulting in both hypertrophic and hypertensive responses, many of which are through transcription factor NFκB activation, ultimately resulting in alterations of gene transcription and perpetuation of the hypertensive state (Gupta, Young et al. 2005; Sriramula, Haque et al. 2008). In order for transcription factors such as NFκB to activate their target genes, DNA and chromatin remodeling must occur. Post-translational modifications of histone cores through a tightly regulated addition/removal of an acetyl tag on their N-terminal tails plays a major role in gene expression modulation (de Ruijter, van Gennip et al. 2003). These additions/removals are accomplished by several members of the histone acetyltransferase (HAT) and histone deacetylase (HDAC) families, which either open or close DNA strands to the actions of transcription factors (Forsberg and Bresnick 2001).

Normally, this balance is tightly controlled, but during conditions of stress and inflammation, activation of PICs can result in increased HDAC activation and histone

acetylation, correlating with an increase in NFκB activity and further increases in PIC expression, including tumor necrosis factor-α (TNF) and the interleukins (ILs) (Keslacy, Tliba et al. 2007; Kim, Rowe et al. 2007). Though HDACs would appear to repress inflammatory responses through reduced gene expression, this view is too simplistic in regards to their non-histone protein acetylation/deacetylation abilities, often times having quite opposite effects in regards to the inflammatory response (Chen, Fischle et al. 2001; Chen, Weng et al. 2005; Kong, Tannous et al. 2006; Bush and McKinsey 2009).

Recent evidence indicates that the various HDAC classes respond differently towards inducing cardiac hypertrophy in non-hypertensive animal models (Zhang, McKinsey et al. 2002; Kong, Tannous et al. 2006; Lee, Lin et al. 2007) and that global HDAC inhibition (HDACi) can prevent these hypertrophic changes (Davis, Pillai et al. 2005). However, it is not known whether HDACi protects against cardiac hypertrophy and hypertensive response by modulating PICs and oxidative stress in spontaneously hypertensive (SHR) rats. A previous study used valproate, a derivative of valproic acid (VPA), to study hypertension in SHR rats. Though they showed a reduction in systolic blood pressure (Sasaki, Nakata et al. 1990), the treatment was only carried out to 9 weeks of age, which is still 3 weeks too young to display the full systemic changes associated with hypertension in this animal model, including cardiac hypertrophy and inflammation. This study was established to assess the role of HDAC blockade on the inflammatory response and its effect on the pathogenesis of hypertension. Therefore, we hypothesize that chronic HDACi will attenuate the inflammatory and hypertensive responses associated with the hypertensive state. To test this, we administered VPA, a fairly novel HDAC inhibitor (Gottlicher, Minucci et al. 2001), especially Class I HDACs, as a long-term treatment in

SHR rats, for assessment of inflammatory, hypertrophic and hypertensive changes associated with essential hypertension.

MATERIALS AND METHODS

All the procedures in this study were approved by the Louisiana State University Institutional Animal Care and Use Committee and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Animals. Adult male SHR or WKY rats were used for this study. They were housed in temperature- (23 ± 2°C) and light-controlled (lights on between 7 AM and 7 PM) animal quarters and were provided with chow *ad libitum*. Vehicle (water) or valproic acid [VPA, 0.71% wt/vol (Kook, Lepore et al. 2003), Sigma] dissolved in water were prepared and provided daily. Another subset of animals (WKY and SHR; n=5 each) were administered hydralazine [HYD, 25mg/kg/day in drinking water (Gupta, Young et al. 2005), Sigma], a direct smooth muscle relaxant and vasodilator, to determine the pressure-independent effect of VPA on cardiac hypertrophy. The experimental procedures were approved by the Louisiana State University Institutional Animal Care and Use Committee in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Experimental Protocol. SHR (n=20) and WKY (n=20) rats underwent echocardiographic assessment at the start and end of the treatment period. Rats received drug or vehicle treatment for 20 weeks starting at 7 weeks of age. Rats were euthanized at 27 weeks of age. Wet heart and lung weights were measured and analyzed against body weight. Blood and left ventricular tissue (LV) was collected for molecular analysis.

Blood Pressure Measurement. A tail-cuff plethysmograph (CODA 6 Blood Pressure System, Kent Scientific System, Torrington, CT) was used for blood pressure measurement at baseline,

and weekly thereafter. Blood pressure was measured for four consecutive days for determination of weekly average measures. The daily measurements were blindly analyzed for the most closely associated five consecutive runs for use as that day's average value.

Echocardiographic Assessment of LV Hypertrophy. Echocardiography was obtained at baseline, and repeated at the end of the treatment period. Echocardiogram was performed as described previously (Mariappan, Elks et al. 2009). Briefly, transthoracic echocardiography was performed under isoflurane anesthesia, using a Toshiba Aplio SSH770 (Toshiba Medical, Tustin, California) fitted with a PST 65A sector scanner (8 MHz probe) which generates two-dimensional images at a frame rate ranging from 300-500 frames per second. Left ventricular posterior wall thickness at end-systole and end-diastole (LVPWTs and LVPWTd, respectively) was measured digitally on M-mode recordings and averaged from at least three cardiac cycles.

Assessment of LV Hypertrophy Through Fibrosis Staining. Paraffin sections (10μm) were obtained from heart specimens as previously described (Sriramula, Haque et al. 2008) and stained with picrosirius red for the detection of collagen. The percent area of fibrosis was calculated using ImageJ software (NIH).

Detection of Total ROS and Superoxide ($O_2^{\bullet-}$) in LV Heart Tissue. One of the most sensitive and definitive methods of superoxide production is electron spin resonance (ESR). In this study, we utilized an established technique for total ROS detection in tissue using ESR and spin traps (Mariappan, Elks et al. 2009). Different spin probes were used for the ESR studies. 1-hydroxy-3-methoxycarbonyl-2, 2, 5, 5-tetramethylpyrrolidine (CMH) was used to measure the $O_2^{\bullet-}$ levels. All ESR measurements were performed using an EMX ESR eScan BenchTop spectrometer and super-high quality factor (Q) microwave cavity (Bruker Company, Germany). **Sample Preparation for ESR studies:** The dissected LV tissue from each animal was placed into a 24-

well plate containing Kreb's HEPES buffer (KHB) (20mM, pH 7.4). Tissue pieces were then washed twice with the same buffer to remove any trace contamination. Samples were then incubated at 37^{o}C with specific spin probes for 30 minutes. **Total tissue ROS production:** Total ROS was determined as previously described (Mariappan, Elks et al. 2009). Tissue pieces were incubated at 37^{o}C with CMH (200 μM) for 30 minutes. Aliquots of the incubated probe media were then taken in 50 μl glass capillary tubes (Noxygen Science Transfer and Diagnostics, Elzach, Germany) for determination of total ROS production, under the following ESR settings: field sweep 50 G; microwave frequency 9.78 GHz; microwave power 20 mW; modulation amplitude 2 G; conversion time 327 ms; time constant 655 ms; receiver gain 1 x 10^5. For superoxide production, samples were pre-incubated at 37^{o}C with PEG-SOD (50 U/ml) for 30 minutes, then CMH (200uM) for an additional 30 minutes. Aliquots of the incubated probe media were taken in 50 μl glass capillary tubes for determination of total superoxide production. Addition of PEG-SOD to CMH allowed competitive inhibition of the $O_2^{\bullet-}$-CMH oxidation reaction by the quenching of $O_2^{\bullet-}$ radicals. Since it is cell permeable, PEG-SOD can competitively inhibit the CMH-$O_2^{\bullet-}$ interaction both intracellularly and extracellularly, thus allowing accurate measurement of total tissue $O_2^{\bullet-}$ production. To determine actual total tissue superoxide production, the values obtained from incubation with PEG-SOD and CMH were subtracted from the values obtained from incubation with CMH only.

RNA Isolation and Real-Time RT-PCR. Total RNA was extracted from the LV using TRI reagent (Invitrogen), and cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad) as previously described (Guggilam, Haque et al. 2007; Sriramula, Haque et al. 2008). The mRNA expression levels of ANP, Collagen IV, TNF, IL-1β, IL-6, the p50 subunit of NF-κB, gp91*phox* and angiotensin II-Type 1 receptor (AT_1R) were determined using previously published specific

custom made primers (Guggilam, Haque et al. 2007; Sriramula, Haque et al. 2008; Agarwal, Haque et al. 2009; Elks, Mariappan et al. 2009; Mariappan, Elks et al. 2009). GAPDH was used as the housekeeping gene. Real-time RT-PCR (qRT-PCR) was performed in 384 well PCR plates using Bio-Rad PCR Master Mix (The iTaq SYBR™ Green Supermix with ROX) and the ABI Prism 7900 sequence detection system (Applied Biosystems). The PCR cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles (15 s at 95°C, 1 min, at 60°C). A dissociation step (15 s at 95°C, 15 s, at 60°C and 15 s at 95°C) was added to check the melting temperature of the specific PCR product.

HDAC Activity Analysis by Colorimetric Assay. Nuclear extracts of LV tissue were obtained with a Nuclear Extraction Kit (BioVision). Nuclear extract was then analyzed for HDAC activity with a Colorimetric HDAC Activity Assay Kit (BioVision), both according to manufacturer's instructions. The plate was read by a Multiskan Spectrum system and values were determined as O.D./μg protein in samples.

AT_1R Protein Analysis by Western Blot. Protein was extracted from LV samples in ice-cold buffer (10mM Tris-HCl, pH 7.4, 1mM EDTA, 1% Nonidet P-40, 0.1% sodium deoxycholate, and 0.1% SDS) containing a protease inhibitor cocktail (Roche). The protein content in the supernatant was determined using a detergent-compatible protein assay (Bio-Rad). Protein samples (25μg) were resolved in 10% SDS-polyacrylamide gel.The protein expression of AT_1R in the heart was analyzed by Western blot as previously described (Sriramula, Haque et al. 2008) with the use of anti-AT_1R antibody (Santa Cruz). Bands were normalized to GAPDH.

Localization of TNF and IL-1β by Immunohistochemistry. Heart tissues were prepared as previously described (Guggilam, Haque et al. 2007). The sections were treated with respective

primary antibodies TNF (1:100 dilution, anti-goat) and IL-1β (1:50 dilution, anti-rabbit) (Santa Cruz). Negative control sections were incubated with secondary antibody alone.

Localization of gp91*phox* by Immunofluorescence. For detection of gp91*phox* in LV heart tissue, slides were incubated overnight at 4ºC with a 1:100 dilution of goat polyclonal anti-gp91*phox* (Santa Cruz) as previously described (Khaleduzzaman, Francis et al. 2007).

Electrophoretic Mobility Shift Assay (EMSA) for Assessment of NF-kB Activity. EMSA was used to assess the activity of NF-kB (p65) in the left ventricle as previously described (Agarwal, Haque et al. 2009).

Statistical Analysis of Data. All results are expressed as mean ± SEM. For statistical analysis of the data, student's *t* test, one-way ANOVA or repeated measures ANOVA followed by Bonferroni's *post hoc* test was performed using GraphPad Prism version 5.0 for Windows, GraphPad Software, San Diego California, USA, to determine differences among groups. Cardiac fibrosis measurements were compared by using nonparametric Kruskal-Wallis ANOVA followed by Mann-Whitney U *post hoc.* A value of $p<0.05$ was considered significant.

RESULTS

VPA Treatment Attenuates the Blood Pressure Changes in SHR Rats. Blood pressure recordings show that SHR+VPA maintained a lower blood pressure level from the pre-hypertensive to the more advanced hypertensive phases as compared to SHR controls (Figure 3.1). Following 10 weeks of treatment (started at 7 weeks of age), SHR control MAP continued to rise while SHR+VPA rats plateaued at a lower pressure (163.1±2.32 vs. 127.5±2.35mmHg, respectively, $p<0.05$). The MAP of SHR+VPA rose slightly throughout the course of the study, becoming significantly elevated above WKY controls towards the end of the treatment period (141.2±4.5 vs. 114.8±3.41, respectively, $p<0.05$), but still significantly lower than the MAP of

SHR controls (141.2±4.5 vs. 173.2±3.47, respectively, $p<0.05$). Mean diastolic and systolic pressures followed the same pattern as the MAP (data not shown), indicating that VPA attenuated all phases of blood pressure in SHR rats. Furthermore, VPA did not have any adverse effects on the health of the animals used in this study.

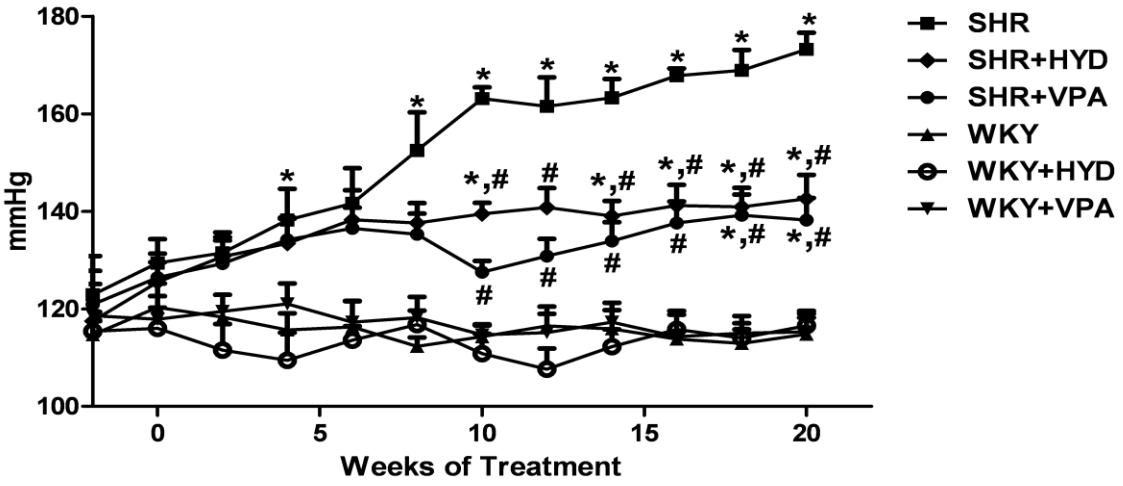

Figure 3.1. Effects of VPA and HYD treatment on MAP. VPA and HYD attenuated the increase in MAP seen in untreated SHR rats. n=5-8 in each group, *$p<0.05$ vs WKY, #$p<0.05$ vs SHR.

Another set of animals (n=5 for each SHR and WKY) were treated with HYD, a direct smooth muscle relaxant and vasodilator, to compare their effects against treatment with VPA (Figure 3.1). SHR+HYD saw a decrease similar to SHR+VPA in MAP as compared to SHR (142.57±4.906 vs. 173.2±3.47). WKY+HYD had no change in MAP. Mean diastolic and systolic followed the same pattern as the MAP in HYD treated rats (data not shown), indicating that HYD similarly attenuated all phases of increased blood pressure seen in SHR rats.

VPA Attenuates Cardiac Hypertrophy in SHR Rats. Echocardiographic assessment showed that SHR controls had significantly more concentric hypertrophy of the left ventricular posterior wall (LVPWT) during diastole and systole (Figure 3.2A) (2.48±0.11 and 3.43±0.2mm,

respectively) when compared to WKY controls (1.73±0.02 and 2.32±0.06, respectively). However, SHR+VPA (1.85±0.03, 2.52±0.04mm) rats displayed no change in ventricular thickness as compared to WKY and WKY+VPA (1.75±0.06, 2.28±0.07mm) rats, indicating that VPA had a beneficial effect on preventing the increased LVPWTd/s that is typically observed in SHR rats.

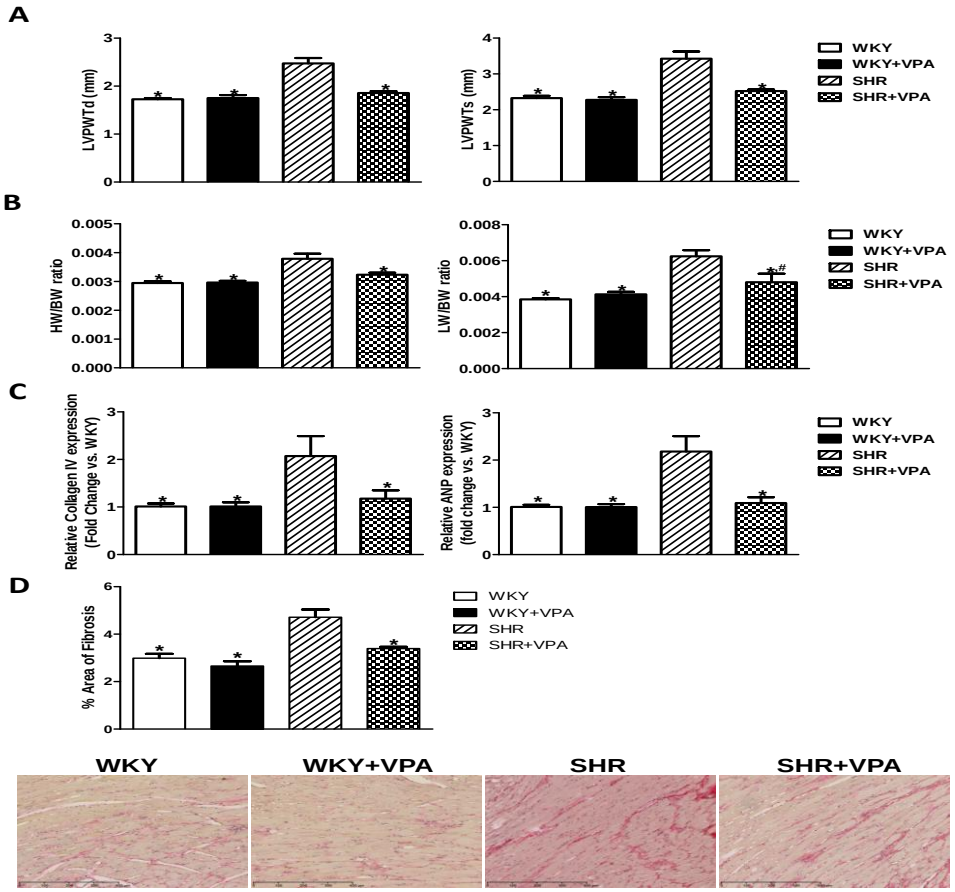

Figure 3.2. Effects of VPA treatment on cardiac hypertrophy. VPA attenuated ventricular wall thickness as assessed by echocardiography (**A**) during both diastole and systole. VPA also attenuated the HW/BW (indicating reduction in cardiac hypertrophy) and LW/BW (indicating reduction in lung edema due to reduced cardiac function) ratio in SHR rats (**B**). SHR rats had increased levels of ANP and Collagen IV mRNA expression which were reduced with VPA treatment (**C**). Graphs expressed as fold change versus WKY rats. VPA reduced cardiac fibrosis versus SHR rats as indicated by picrosirius red staining collagen versus total area (**D**). Stained slides are representative of typical results for each group (n=3/group). n=7-8 in each group, *p<0.05 vs WKY, #p<0.05 vs SHR.

Heart weight (HW)/body weight (BW) and lung weight (LW)/BW ratios are often used to show phenotypic changes due to hypertension such as increased heart mass due to hypertrophy (HW/BW) and increased edema of the lungs (LW/BW) due to cardiac dysfunction and increased systemic circulatory resistance. Treatment with VPA or HYD alone did not have any effects on BW (Figure 3.3A). SHR+VPA normalized both HW/BW and LW/BW indices versus SHR control rats (0.0032 and 0.0048 vs. 0.0039 and 0.0062, respectively, $p<0.05$) as compared to WKY (0.0029 and 0.0038), reinforcing that VPA reduces cardiac hypertrophy and dysfunction (Figure 3.2B). However, the HW/BW ratio assessed for SHR+HYD did not improve cardiac hypertrophy (Figure 3.3B) as compared to SHR controls (0.0036 vs. 0.0039), indicating that improvements in cardiac hypertrophy due to VPA treatment were not pressure dependent.

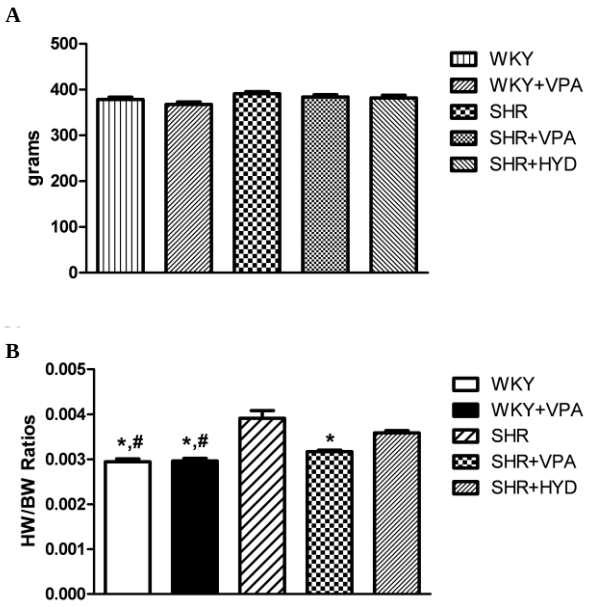

Figure 3.3. Effects on VPA and HYD treatment on BW and cardiac hypertrophy. There were no BW differences across SHR and WKY rats or between treatment groups. VPA attenuated the HW/BW (indicating reduction in cardiac hypertrophy) ratio, whereas HYD had minimal effect in reducing cardiac hypertrophy in SHR rats. n=7-8 in each group, *$p<0.05$ vs WKY, #$p<0.05$ vs SHR.

VPA Reduces Hypertrophic Response Elements in the LV Tissue of SHR Rats. To further demonstrate the effects of VPA on hypertrophy, RT-PCR analysis was undertaken on hypertrophic response genes in the LV. Compared to WKY, untreated SHR rats had elevated levels of collagen IV and atrial natriuretic peptide (ANP) - two markers of LV remodeling

associated with cardiac hypertrophy (Figure 3.2C), as well AT_1R. SHR+VPA reduced these levels to that of normotensive WKY controls ($p<0.05$), indicating HDACi has a positive effect on reducing molecular markers of cardiomyocyte and interstitial growth normally attributed to systemic hypertension. Furthermore, SHR+VPA had reduced % fibrosis staining (Figure 3.2D) as compared to SHR rats (3.395±0.07 vs 4.713±0.32), signifying a reduction in total fibrosis within the heart following HDACi. The mRNA expression of AT_1R was significantly increased within the LV of SHR rats versus that of normotensive WKY controls (2.7±0.5 fold vs WKY) (Figure 3.4B), which was fully attenuated in SHR+VPA rats (2.7±0.5 vs. 0.9±0.1 fold vs WKY, respectively) and reconfirmed by western blot of the LV tissues (Figure 3.4A). HYD had no effect on the mRNA expression of either ANP or AT_1R (Figure 3.5A, B), demonstrating the effect that HDACi has on controlling these locally activated hypertrophic mediators in the LV of SHR rats.

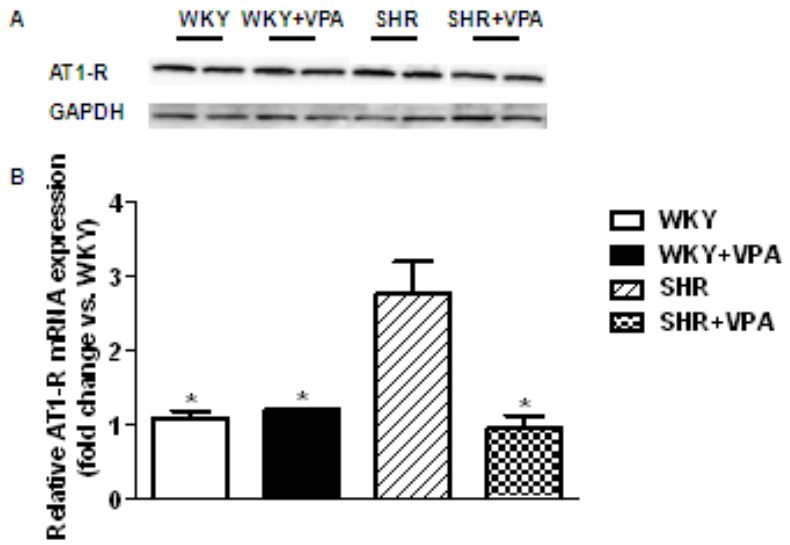

Figure 3.4. Effect of VPA on AT_1R mRNA and protein expression. Untreated SHR rats showed higher mRNA (**B**) and protein expression (**A**) levels of the AT_1R in the LV when compared to WKY controls. SHR+VPA attenuated this increase. Western blot protein expression bands were normalized to GAPDH. n=7-8 in each group, *$p<0.05$ vs SHR.

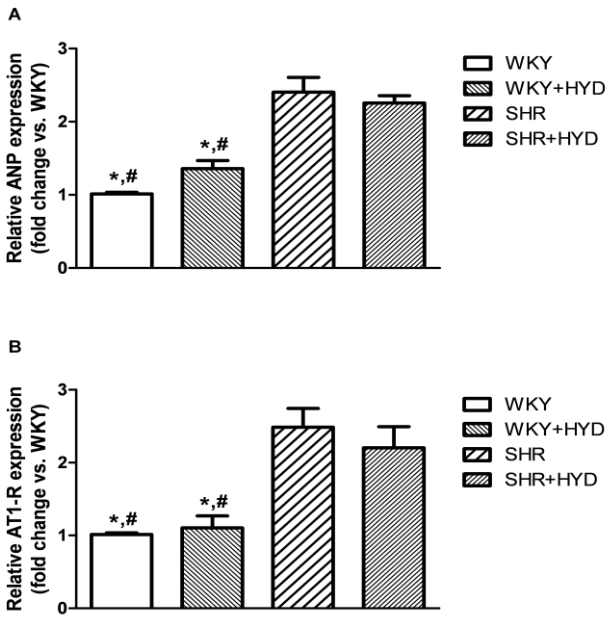

Figure 3.5. Effects of HYD treatment on hypertrophic markers. SHR rats had increased levels of ANP (**A**) and AT1-R (**B**) mRNA expression compared to WKY controls. These levels remained unchanged following HYD treatment. n=5-8 in each group, *p<0.05 vs SHR, #p<0.05 vs SHR+HYD.

VPA Reduces HDAC Activity in the LV of Treated Groups. To assess the effectiveness of VPA on HDAC activity in LV tissue, a colorimetric assay kit was used to analyze differences between the VPA treated and untreated groups (Figure 3.6A). Global HDAC activity was reduced in untreated WKY versus untreated SHR rats (17.87±1.06 vs. 22.19±0.57 O.D./mg protein sample, respectively, p<0.05). Furthermore, WKY+VPA and SHR+VPA groups both exhibited a lower HDAC activity level (not significant and p<0.05, respectively) as compared to their own strain controls. Conversely, SHR and WKY rats treated with HYD did not have a decrease in HDAC activity when compared with their respective controls (data not shown).

VPA Normalizes Inflammatory Response in the LV Tissue of SHR Rats. Immuno-histochemistry revealed that SHR controls had increased protein expression of TNF and IL-1β in the LV as compared to WKY controls. This protein expression was reduced in SHR+VPA rats (Figure 3.7A). RT-PCR similarly indicated that SHR controls had increased expression of TNF, IL-1β, IL-6 (Figure 3.7B) mRNA and NFκB p65 (Figure 3.6B, C) activity and mRNA in LV tissue when compared to WKY controls. This increase was attenuated in SHR+VPA rats, while WKY+VPA exhibited no change. Furthermore, HYD had no effect on the PIC mRNA

expression of TNF or IL-1β (data not shown). These combined results indicate that VPA treatment reduces and normalizes the inflammatory response observed in SHR rats.

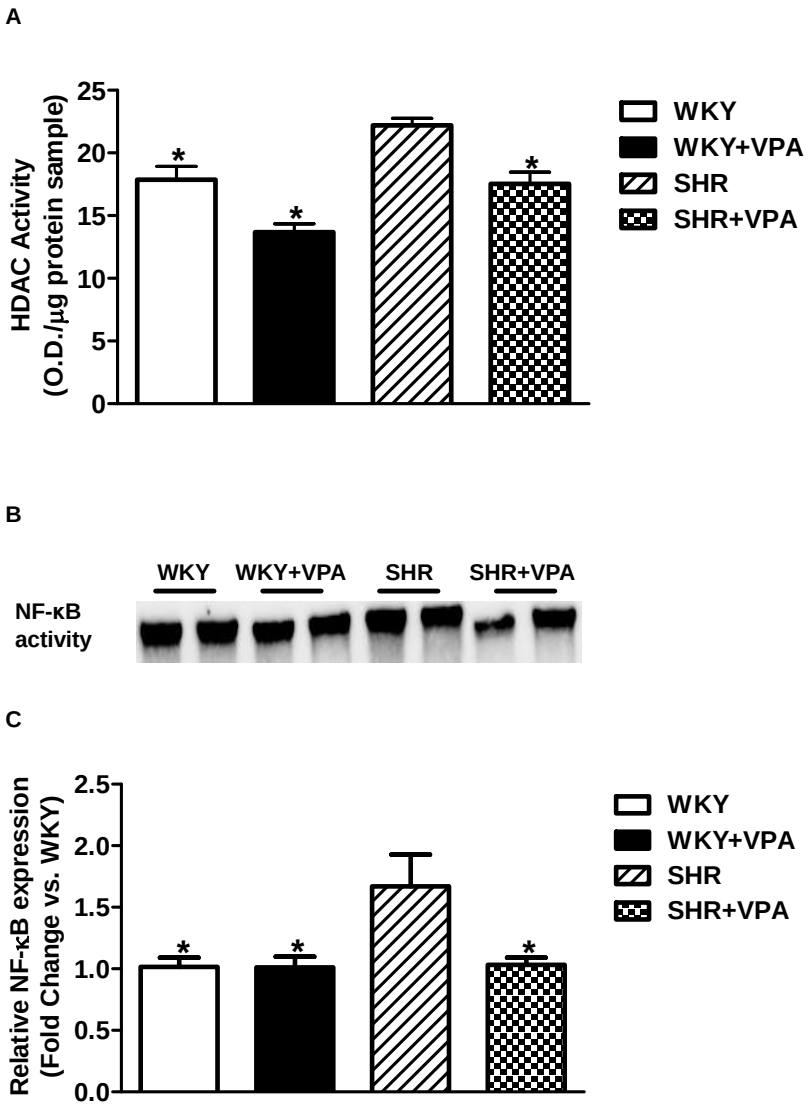

Figure 3.6. Effects of VPA on HDAC activity as assessed through a colorimetric detection assay and NFκB (p65) activity and expression via EMSA and real-time RT-PCR, respectively, in the LV. HDAC activity was elevated in both WKY and SHR rats versus WKY+VPA and SHR+VPA rats (**A**). Untreated SHR HDAC activity was elevated versus untreated WKY rats. Untreated SHR rats had an increased activity (as determined by EMSA) and mRNA expression of NF-κB which was attenuated in SHR+VPA (**B, C**). n=7-8 in each group, *p<0.05 vs SHR.

VPA Reduces ROS and gp91*phox* in the LV of SHR Rats. As inflammation during hypertensive response is associated with an increase in oxidative stress, both total ROS as assessed by EPR (Figure 3.8A) and the expression of gp91*phox* (Figure 3.8B, 6C) were examined in the LV. Untreated SHR rats experienced a significant increase in ROS when compared to WKY rats (0.54±0.05 vs. 0.22±0.03mM/mg protein/min). SHR+VPA normalized this ROS increase (0.54±0.05 vs. 0.21±0.03mM/mg protein/min). Furthermore, gp91*phox*, the major catalytic subunit of NADPH oxidase and a ROS contributor, was increased in untreated SHR rats versus WKY controls. This was subsequently reduced in SHR+VPA (3.39 vs. 1.56 fold change/WKY), but not SHR+HYD (data not shown). The reduction in protein expression was confirmed by immunofluorescence of the LV tissue. These results indicate that treatment of SHR rats with VPA had beneficial effects in reducing oxidative stress in the LV tissue.

DISCUSSION

The major findings in this study are as follows: 1) HDACs played an important role in hypertensive drive by modulating inflammatory and oxidative stress actions and contributed to hypertrophic and hypertensive responses in SHR rats, 2) HDACi attenuated MAP in SHR rats, 3) HDACi also attenuated LVPWT and HW/BW ratio in SHR rats, demonstrating that HDACi reduces cardiac hypertrophy, possibly in part through modulation of ANP, Collagen IV and AT_1R expression, 4) untreated SHR rats had an increase in inflammatory markers, including TNF and NFκB, as well as an increase in ROS and gp91*phox* expression, which were decreased through long-term HDACi using VPA, and 5) HDACi reduced AT_1R expression, thereby limiting the role that angiotensin II (Ang II) plays in mediating hypertension. These findings suggest that long-term treatment with VPA reduces hypertension-induced PICs, thereby attenuating hypertrophic and hypertensive responses in SHR rats.

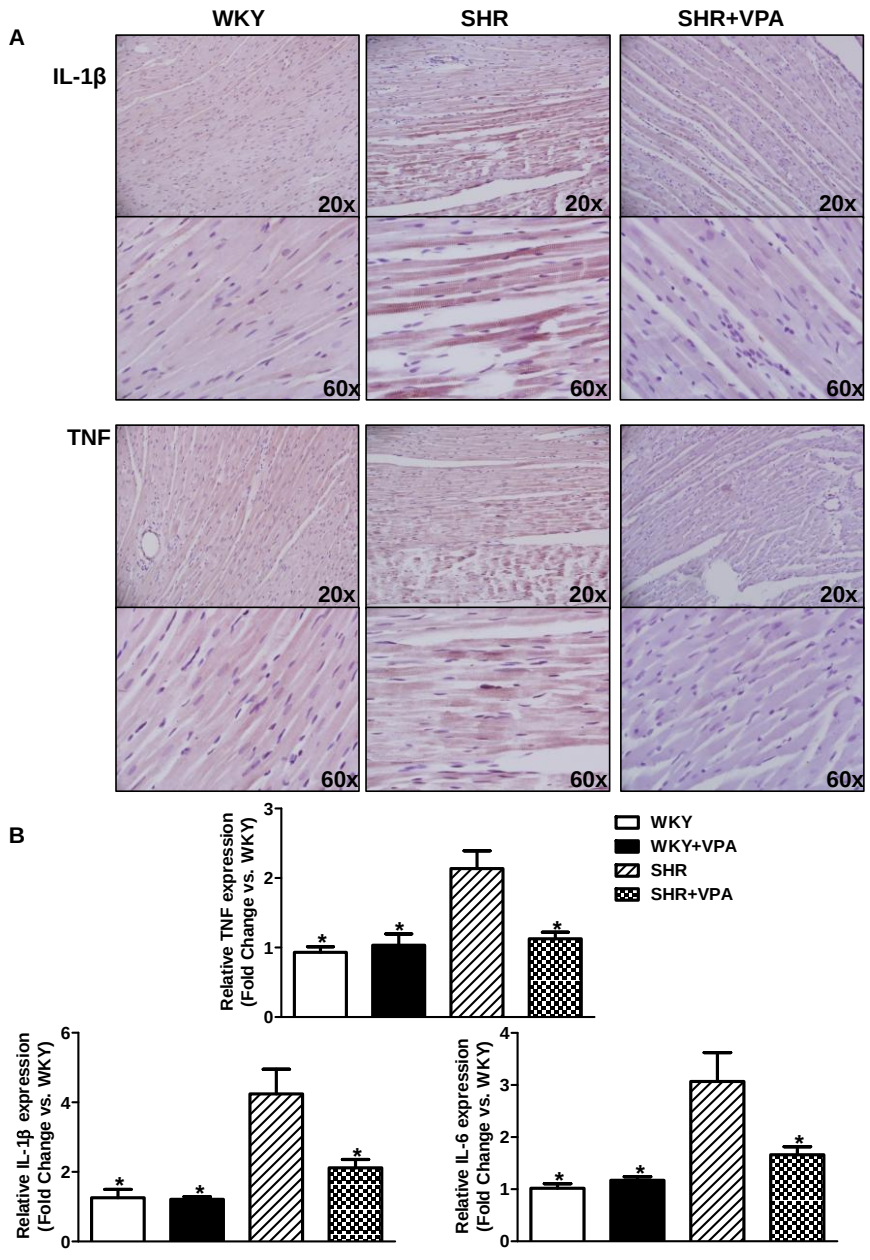

Figure 3.7. Effects of VPA treatment on TNF and IL-1β protein and mRNA expression. SHR rats had notably increased protein staining of TNF and IL-1β in the LV when compared to WKY rats as shown through immunohistochemical staining (**A**). This staining increase was markedly reduced in SHR+VPA. Images shown represent results observed in preparations from 4 to 6 rats. Untreated SHR rats had increased levels of PIC expression compared to WKY rats (**B**). SHR+VPA reduced this increased expression in the LV tissue. n=7-8 in each group, **p*<0.05 vs SHR.

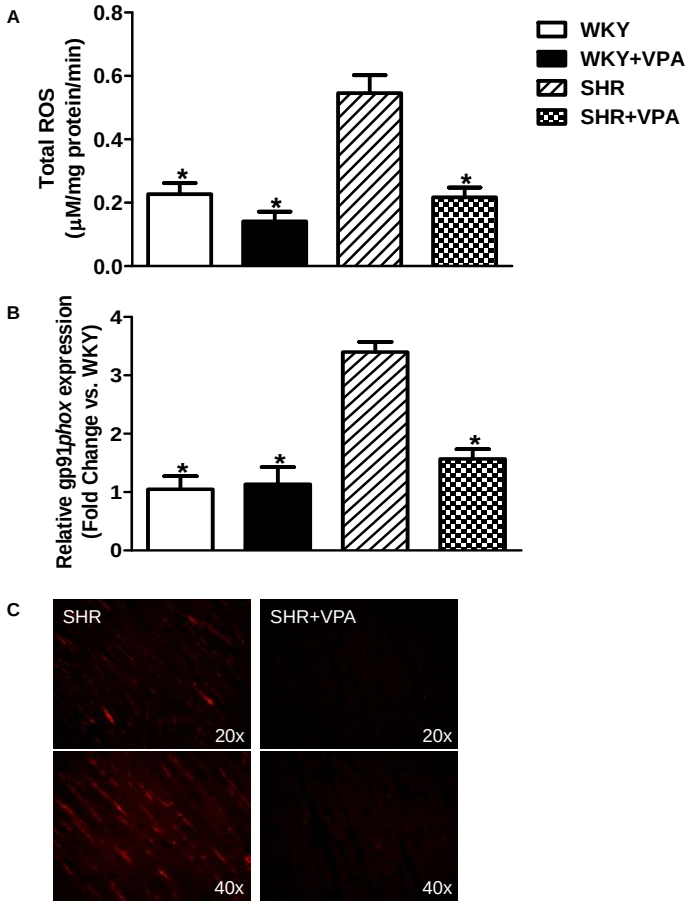

Figure 3.8. Effects of VPA treatment on tROS and gp91*phox* in the LV. SHR rats had increased tROS levels as assessed by EPR. These levels were normalized in SHR+VPA (**A**). SHR rats also showed increased mRNA (**B**) and protein (**C**) expression of gp91*phox*, the catalytic subunit of NADPH oxidase, in the LV versus WKY rats. This was attenuated in SHR+VPA. Immunofluorescence of LV cardiomyocytes shows increased presence of gp91*phox* in SHR rats versus SHR+VPA. n=7-8 in each group, **p*<0.05 vs SHR.

HDACs play an important role in inducing the structural remodeling of chromatin that exposes DNA to transcription factors, ultimately yielding changes in gene expression. Along with HATs, HDACs maintain a relative balance in cellular systems during normal physiology that allows typical function (de Ruijter, van Gennip et al. 2003). However, during diseased conditions, this balance is tilted in favor of HDACs, which can lead to an elevation in inflammatory and immune responses (Wade 2001; Aung, Schroder et al. 2006; Brogdon, Xu et al. 2007; Ito, Charron et al. 2007; Kim, Rowe et al. 2007; Halili, Andrews et al. 2009). In this study, we also observed an elevated HDAC activity which was accompanied by increased PIC and oxidative stress gene expression in the hearts of SHR rats.

Cardiac hypertrophy is a well described consequence of systemic hypertension. Studies have shown the role of HDACi on controlling cardiac growth and remodeling (Antos, McKinsey et al. 2003). However, little is known about the holistic role of HDACi on hypertrophy during hypertensive response. As evidence indicates (Zhang, McKinsey et al. 2002; Antos, McKinsey et al. 2003; Davis, Pillai et al. 2005; Kee, Sohn et al. 2006; Kong, Tannous et al. 2006), pathological cardiac hypertrophy and function rely upon the balanced abundance of α- and β-myosin heavy chain (MHC) protein throughout the heart. During the progression of cardiac hypertrophy, the adult isoform of MHC (α-MHC) undergoes a stressed-trigger switch to the fetal isoform (β-MHC), contributing to cardiac hypertrophy. HDACi blunts MHC isoform switching, therefore preserving ventricular function (Kong, Tannous et al. 2006; Lee, Lin et al. 2007; Bush and McKinsey 2009). These results are contradictory to earlier experiments showing that class II HDACs block pro-growth genes through interaction with transcription factor myocyte enhancer factor-2 (MEF2) (McKinsey, Zhang et al. 2002). More recently though, research has indicated that the pro-hypertrophic class I HDACs, when activated, are more potent and take priority over the anti-hypertrophic class II HDACs (Antos, McKinsey et al. 2003), thus explaining how global HDACi can attenuate cardiac hypertrophy in various animal models (Kee, Sohn et al. 2006; Kong, Tannous et al. 2006). From these changes, it has been suggested that within the hypertrophied heart, hypertrophic stress signals cause the phosphorylation of HDACs bound to MEF2, causing their disassociation into the cytoplasm. Since these HDACs are not bound to the chromatin structure, their inhibition would have no overt effect on MEF2 transcription, indicating that HDACi has no direct effect on the depression of MEF2 controlled pro-hypertrophic genes and signifying that another mechanism must be in place (Kong, Tannous et al. 2006).

HDACi using Trichostatin A, an inhibitor of Class I and II HDACs, either induced (Kuwahara, Saito et al. 2001) or blunted (Antos, McKinsey et al. 2003) agonist-induced expression of ANP, a hypertrophic growth factor, in cultured neonatal myocytes. The present study also demonstrated that HDACi in SHR rats showed a significant reduction in ANP, suggesting that in SHR rats, ANP possibly plays an important role in attenuating cardiac hypertrophy, and that HDACi silences/blunts this signaling mechanism. Moreover, collagen IV, an indicator of cardiac remodeling, especially within the failing heart (Grimm, Huber et al. 2001), was increased in SHR rats, but not SHR+VPA rats. Finally, AT_1R, an important component of the RAS which, when acted upon by Ang II in the LV, causes cardiac hypertrophy (Makino, Sugano et al. 1997), has been shown to be attenuated with VPA treatment (Kee, Sohn et al. 2006; Lu and Yang 2009), but the mechanism involved has not been fully investigated. The present study showed significantly reduced AT_1R expression in SHR+VPA rats versus SHR controls, indicating that a possible hypertrophic mechanism intimately involves AT_1R activation.

There are several well known modulators of pressure-independent cardiac hypertrophy including Ang II and sympathetic neurohormones (Kang, Ma et al. 2009). Recently, it was also shown that Ang II-induced cardiac hypertrophy can be prevented using HDACi (Kee, Sohn et al. 2006). To determine if the current study's effects on cardiac hypertrophy were pressure-independent or -dependent of HDACi, we looked at the effect of HYD on blood pressure and cardiac hypertrophy, as well as on ANP and AT_1R expression. HYD reduced MAP in SHR rats similar to VPA; however, cardiac hypertrophy was unaffected, including hypertrophic mediators ANP and AT_1R. This indicates a possible pressure-independent mechanism regarding VPA's effect on cardiac hypertrophy. These results are in agreement with a recent study where NFκB inhibition reduces cardiac hypertrophy in a pressure-independent manner (Gupta, Young et al.

2005). This may offer another mechanism whereby HDACi reduces cardiac hypertrophy, for results herein show a decrease in NFκB activity and expression following VPA treatment. Therefore, from these results, not only was cardiac hypertrophy reduced through long-term VPA treatment, but several possible mechanisms that induce cardiac remodeling were also attenuated.

We and others have recently demonstrated that hypertensive drive is partially controlled through the over-expression of PICs, especially TNF, along with downstream alterations in NFκB, ROS and RAS components, as regulated through AT_1R activation (Wilcox and Welch 2001; Sriramula, Haque et al. 2008; Elks, Mariappan et al. 2009; Mariappan, Elks et al. 2009). This study demonstrates that chronic HDACi attenuates PIC response in SHR+VPA rats. Moreover, VPA reduces the presence of ROS and AT_1R, two components implicated in the inflammatory response observed in hypertension. HDACi has been increasingly identified as a possible therapeutic approach towards many inflammatory conditions, including cardiovascular diseases (Gottlicher, Minucci et al. 2001; Adcock 2007; Bush and McKinsey 2009; Halili, Andrews et al. 2009). Though this mechanism has not yet been entirely delineated, it is suggested that the use of a HDACi, such as VPA, blocks HDAC actions on protein function outside of its normal action on altering transcription (Chen, Weng et al. 2005; Bode, Schroder et al. 2007), as subsets of these families possess the ability to act on non-histone proteins, further complicating their roles in gene modulation (Walkinshaw, Tahmasebi et al. 2008). A recent study showed that HDACi can deactivate Akt, a potential mediator of cardiac hypertrophy and oxidative stress, via dephosphorylation by HDAC-protein phosphatase 1 complexes (Chen, Weng et al. 2005). However, other studies have shown that specific sets of Toll-like receptor-inducible genes are targeted by HDACi in macrophages and dendritic cells, and that one of these is through the NFκB pathway (Aung, Schroder et al. 2006; Brogdon, Xu et al. 2007), which

concurs with the present study. It underscores the role of HDACi on PIC activation of NFκB, preventing a further, cyclically driven up-regulation of PICs, including TNF, IL-1β and IL-6.

The effect of inflammation on ROS in hypertension has been previously demonstrated (Paravicini and Touyz 2006; Elks, Mariappan et al. 2009). A number of pro-inflammatory mediators of this increased ROS have been identified, including TNF and IL-6, which, as demonstrated here, are attenuated with HDACi, confirming the roles of HDACs on inflammatory and oxidative stress responses on hypertension in SHR rats. Though the signaling pathway is not entirely clear on how HDACi attenuates ROS in SHR rats, either directly or indirectly through its blockade of PICs and the NFκB pathway, we postulate that by blocking PIC activation, with its subsequent down-regulation of NFκB activity and gp91*phox* expression, ROS is inhibited.

The interaction between the RAS, PICs and ROS has also been demonstrated by work in our lab (Sriramula, Haque et al. 2008) and others (Brasier, Li et al. 1996; Sasamura, Nakazato et al. 1997; Arenas, Xu et al. 2004). Presently we show that untreated SHR rats have increased AT_1R expression, an important component of the pro-hypertensive portion of the RAS. HDACi through VPA treatment attenuated this increase concomitant with that of PICs. The pathophysiological mechanism of hypertension intimately involves the action of Ang II, including vasoconstriction, increased aldosterone secretion, increased sympathetic nerve activity, tissue remodeling and increased sodium and water intake, all of which are mediated through AT_1Rs that are distributed throughout most organ systems, including the liver, brain, kidney, heart and blood vessels (Allen, Zhuo et al. 2000). Reports indicate that Ang II is controlled by, and controls, HDAC-induced changes in gene and protein response (Kee, Sohn et al. 2006; Lu and Yang 2009). Here we show a possible new mechanism involving the regulation of Ang II responses as directed through the AT_1R. HDACi reduces AT_1R gene expression and receptor

density, thereby ameliorating the actions of Ang II. This alteration in AT_1R expression could be either through direct HDACi effects on the receptor's production and function, or through the effects of HDACi on inflammatory gene response during hypertension. This mechanism must be further investigated in order to determine the full effect of HDACi in attenuating hypertension.

In conclusion, the present study's results show that long-term HDACi through VPA attenuated MAP and cardiac hypertrophy, possibly through modulation of ANP, Collagen IV and AT_1R. HDAC inhibition also attenuated the increased inflammatory response, including TNF and NFκB, as well as the increase in ROS and gp91*phox*. These findings suggest that HDACi with VPA reduced inflammation, ROS, and AT_1R, thereby attenuating hypertension and its secondary consequences in SHR rats.

PERSPECTIVES

We chose to use VPA due to its current use in clinical settings as an anti-seizure and bipolar drug, demonstrating its availability to patients. In our study, VPA was administered long-term without any adverse effects towards the treated animal groups. This outlines the importance of the continuous drug administration necessary for the successful treatment of hypertension and its consequences, including cardiac hypertrophy, systemic inflammation and end organ damage due to ROS. While we cannot rule out the effects of VPA on blood pressure from its GABAergic actions or non-histone protein interactions, we feel confident that this study provides sufficient evidence that the use of HDACi can reduce not only blood pressure, but cardiac hypertrophy and the inflammatory state associated with hypertension. The specific mechanisms involved in HDACi must be studied more closely. However, as the quest to find new therapeutic strategies in hypertensive control is ever pressing, this could present a possible new approach in future treatment options.

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CHAPTER 4

CENTRAL TUMOR NECROSIS FACTOR-α BLOCKADE REVERSES ANGIOTENSIN II-INDUCED ALTERATIONS IN RENIN-ANGIOTENSIN SYSTEM COMPONENTS AND ATTENUATES HYPERTENSION

INTRODUCTION

It is well known that the renin-angiotensin system (RAS) plays a major role in blood pressure regulation and the hypertensive condition. While the pro-hypertensive components of the RAS have been extensively investigated, the recent identification of an anti-hypertensive set of RAS components has complicated the manner by which the fully activated RAS functions in physiological and pathophysiological states. Angiotensin II (Ang II), the effector peptide of the pro-hypertensive axis of the RAS that also includes angiotensin converting enzyme (ACE) and the Ang II-Type 1 receptor (AT_1R), exerts diverse physiological actions in both the peripheral and central neural systems. The anti-hypertensive counterbalance to these mediators includes ACE2, and the AT_2 and Mas receptors. Importantly, all these essential components of the RAS, including renin and angiotensinogen, as well as various cardiovascular-modulatory aminopeptidases, are synthesized within the brain, suggesting the existence of a comprehensive intrinsic brain RAS (Bader and Ganten 2002; Veerasingham and Raizada 2003; Sakai and Sigmund 2005; Xu, Sriramula et al. 2011). Recent evidence suggests that dysregulation of the individual brain RAS arms may play a critical role in the development and maintenance of hypertension. Ang II, acting through the AT_1R, plays a prominent role in the central regulation of blood pressure by activating the sympathetic nervous system, regulating fluid and salt balance and the secretion of aldosterone, amongst other actions (Veerasingham and Raizada 2003). Furthermore, the pressor response of chronic low-dose Ang II infusion in animals has been shown, at least in part, to be sympathetically driven (Bruner and Fink 1986; Gorbea-Oppliger and Fink 1994). Previous studies suggest that systemically delivered Ang II likely acts upon the circumventricular organs, where the blood brain barrier is weak or absent, and subsequently activates hypothalamic and brain stem sites such as the paraventricular nucleus (PVN) and

ventrolateral medulla, contributing to sympathoexcitation and hypertensive response (Simpson 1981; Fink, Bruner et al. 1987; Collister and Hendel 2003).

Experimental evidence indicates that the hypothalamic PVN is an important center for integrating Ang II-induced neural outflow signals for the pressor response and sympathetic vasomotor tone (Martin, Segura et al. 1991; Zhu, Gao et al. 2004). Recent findings from our lab and others suggest that the RAS, in addition to inducing neurohumoral excitation, also increases the production of proinflammatory cytokines (PICs), such as tumor necrosis factor-alpha (TNF), in brain cardiovascular regulatory centers, and has been shown to contribute to the neurogenic component of hypertension, both through direct actions and through modulating reactive oxygen species (ROS) signaling pathways (Kang, Ma et al. 2009; Shi, Diez-Freire et al. 2010). A chronic increase in peripheral Ang II levels is proposed to initiate a cascade of signaling events involving PICs and ROS in brain cardioregulatory sites raising sympathetic activity, hypertension and end organ damage. These observations, coupled with the emerging role of PICs and the little known role of the anti-hypertensive axis of the RAS in hypertension led us to hypothesize that the central effects of Ang II are, at least in part, mediated by the activation of PICs, especially TNF. The resultant of these actions are the differentially dysregulated RAS arms within cardiovascular relevant brain regions, including the PVN, ultimately enhancing the neurogenic hypertensive response. Presently, we investigate this hypothesis by examining central TNF inhibition via intracerebroventricular (ICV) etanercept (ETN) infusion, a soluble TNF receptor fusion protein, on pro- and anti-hypertensive RAS components in the PVN in Ang II-induced hypertension.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (8-10 weeks old) were used in this study. Animals were housed in a temperature-controlled room (25 ± 1^{o}C) and maintained on a 12:12 hour light:dark

cycle with free access to food and water. All animal and experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Louisiana State University in compliance with NIH guidelines.

Experimental Protocol. The rats were implanted with radio-telemetry transmitters to measure blood pressure, and subjected to ICV infusion of etanercept (10μg/kg/day) or artificial cerebrospinal fluid (aCSF) (Alzet, model 1004; 0.11μl/hr), with and without subcutaneous infusion of Ang II (200 ng/kg/min) for 4 weeks. Osmotic minipumps (Alzet, model 2004; 0.25μl/hr) were filled with Ang II dissolved in 0.9% saline or saline alone, and were implanted subcutaneously in the retroscapular area. The rats were divided into 4 groups: 1) Control group - saline minipumps + ICV aCSF, 2) ETN group - saline minipumps + ICV ETN, 3) Ang II group - Ang II minipump + ICV aCSF, and 4) Ang II+ETN group - Ang II minipump + ICV etanercept. At the end of the study, rats were euthanized the brain was removed for further analysis.

Blood Pressure Measurement. Blood pressure was measured continuously in conscious rats implanted with radio-telemetry transmitters (Model TA11PA-C40, Data Sciences International, St. Paul, MN). Rats were anesthetized with a ketamine (90 mg/kg) and xylazine (10 mg/kg) mixture (i.p.) and placed dorsally on a heated surgical table. An incision was made on the ventral surface of the left leg, and the femoral artery and vein were exposed and bluntly dissected apart. The femoral artery was ligated distally, and a small clamp was used to temporarily interrupt the blood flow. The catheter tip was introduced through a small incision in the femoral artery, advanced ~6 cm into the abdominal aorta such that the tip was distal to the origin of the renal arteries, and secured into place. The body of the transmitter was placed into the abdominal cavity and secured to the abdominal wall. The abdominal musculature was sutured and the skin layer was closed following implantation. Rats received benzathine penicillin (30000 U, i.m.) and

buprenorphine (0.1 mg/kg, s.c.) immediately following surgery and 12 h postoperatively and allowed to recover for seven days.

ICV Cannula Implantation. Following the transmitter recovery period, the rats were implanted with ICV cannula for infusion of ETN or aCSF (Francis, Weiss et al. 2003). The rats were anaesthetized and the head was positioned in a Kopf stereotaxic apparatus. An ICV cannula was implanted into the right lateral cerebroventricle (1.3 mm caudal to bregma, 1.5 mm lateral to the midline, and 3.5 mm ventral to the dura) according to Paxinos and Watson, and fixed to the cranium using small screws and dental cement. A 4-week osmotic minipump was implanted subcutaneously and connected to the infusion cannula via the catheter tube to deliver ETN or aCSF into the brain.

Real Time RT-PCR. PVN punches were made from frozen brain sections using a Stoelting brain punch (Stoelting). Total RNA was isolated from PVN tissue using RNeasy plus micro kit (Qiagen) and cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad). Real Time PCR amplification reactions were performed with iQ SYBR Green Super mix with ROX (Bio-Rad) using the ABI Prism 7900 Real time PCR machine (Applied Biosystems). List of primers used was provided in Table 4.1. Data were normalized to GAPDH expression by the $\Delta\Delta C_T$ comparative method.

Immunohistochemical Analysis. Rats (n=5 in each group) were transcardially perfused with 200 ml of ice-cold PBS (7.4 pH; 0.1M) followed immediately by 200 ml of 4% paraformaldehyde in PBS. The brains were removed, post-fixed in 4% paraformaldehyde solution for 2 hours, and transferred to a phosphate buffer containing 20% sucrose (pH 7.4) and stored overnight. For immunostaining, 10 µm coronal sections from paraffin embedded brains were collected on slides. First the sections were incubated with 0.3% H_2O_2 in methanol for 10

minutes. For antigen retrieval, citrate buffer with microwave heating technique is used. Then the sections were incubated with 1.5% goat or rabbit serum in PBS containing 0.3% Triton X100 for 30 minutes. The sections were incubated with primary antibodies overnight at 4°C followed by incubation with biotinylated goat-anti rabbit or rabbit-anti goat secondary antibodies for 60 minutes, and stained with Vectastain ABC kit (Vector Laboratories) according to the manufacturer's instructions. Each step was followed by washing the sections with PBS containing 0.3% Triton X100. Sections incubated without primary antibody were used as negative controls.

Statistical Analysis. All results are expressed as mean±SEM. For statistical analysis of the data, student's *t* test, one-way ANOVA or repeated measures ANOVA followed by Bonferroni's *post hoc* test was performed using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego California, USA) to determine differences among groups. A value of $p<0.05$ was considered statistically.

RESULTS

Effect of Ang II Infusion on the Mean Arterial Pressure. To assess the effect of central TNF blockade on the Ang II-induced hypertensive pressure response, mean arterial pressure (MAP) was measured using a radio-telemetry system (Figure 4.1A). After 28 days, chronic Ang II infusion significantly increased the MAP in rats when compared with control rats (164.9±5.03 mmHg vs 107.7±8.03 mmHg, respectively; $p<0.05$). In contrast, ICV treatment with etanercept attenuated the Ang II-induced increase in MAP (126.4±21.2 mmHg vs 164.9±5.03 mmHg, respectively; $p<0.05$), while ETN treatment alone had no effect on MAP (103.6±3.47 mmHg).

To evaluate Ang II-induced changes on cardiac hypertrophy in these rats, the hearts were harvested and weighed at the end of experimental period. The ratio of heart/body weight

(HW/BW) was calculated as an indicator of cardiac growth. Chronic Ang II infusion lead to increased cardiac mass when compared to controls (4.04±0.2 vs 3.16±0.1 mg/g, respectively; $p<0.05$), as indicated by the increased HW/BW ratio (Figure 4.1B). ICV treatment with ETN inhibited Ang II-mediated cardiac growth (reduced to 3.15±0.1 mg/g; $p<0.05$). This data suggests a role for TNF in the brain on Ang II-induced blood pressure regulation and cardiac hypertrophy in the hypertension state.

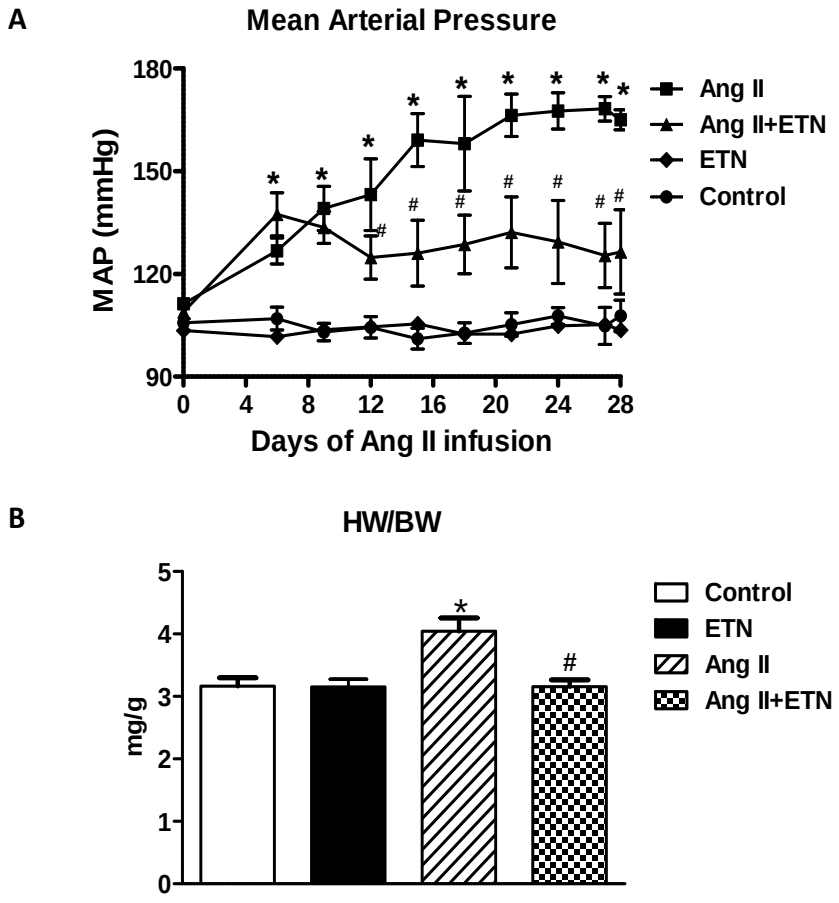

Figure 4.1. Effects of central TNF blockade mean arterial blood pressure (MAP) and cardiac hypertrophy. MAP was increased after 28-day chronic Ang II infusion, this was attenuated in Ang II+ETN rats (**A**). HW/BW ratio as assessed upon sacrifice shows an increase in cardiac mass 28-day Ang II treatments versus controls (**B**). Etanercept reversed this increase in cardiac mass. n=9-10 per group, *$p<0.05$ vs Control, #$p<0.05$ vs Ang II.

Effect of Ang II Infusion on the Expression of PICs in the PVN. To determine the effect of Ang II on the production of PICs and chemokines, the mRNA expression of TNF, IL-6, IL-1β, and the chemokine MCP-1 were measured in the PVN by real time RT-PCR (Figure 4.2). Ang II infusion induced an increase in the gene expression of the PICs TNF, IL-6 and IL-1β, and the chemokine MCP-1, in the PVN, when compared to saline and saline+ETN control rats. These PICs were attenuated in the PVN of Ang II-infused rats treated ICV with ETN, demonstrating that chronic Ang II infusion increases the pro-inflammatory response within the PVN through TNF in Ang II-induced hypertension.

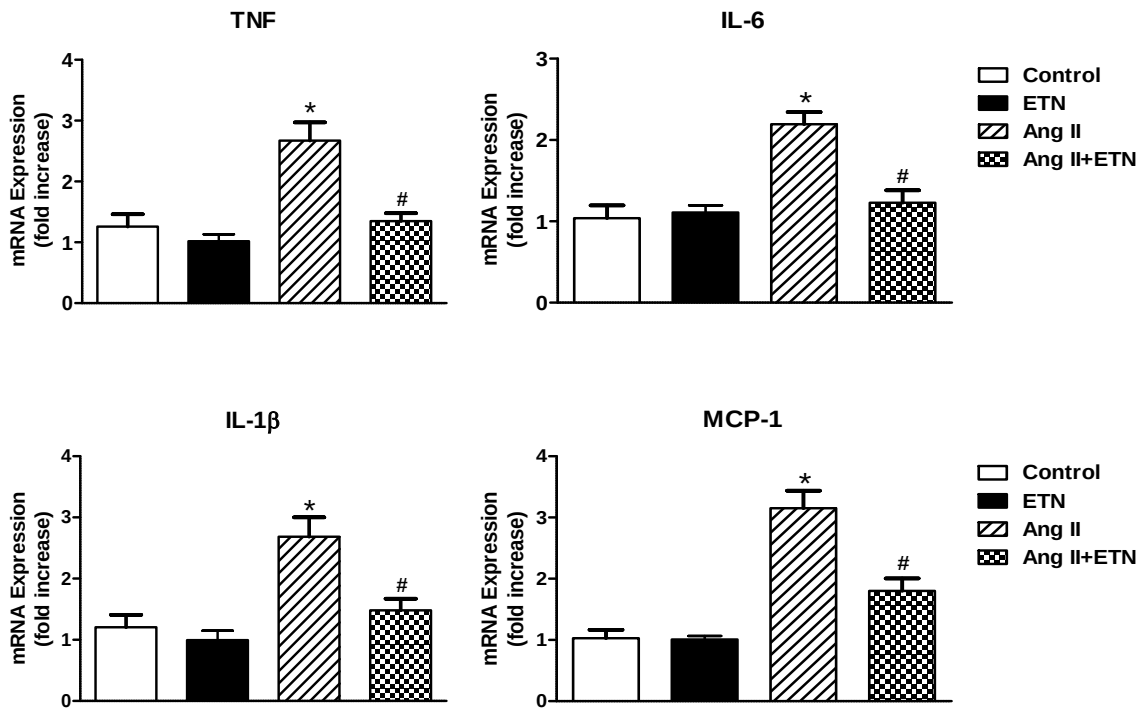

Figure 4.2. Effects of central TNF blockade PIC and chemokine expression in the PVN. Chronic Ang II infusion increased the expression of TNF, IL-1β, IL-6 and the chemokine MCP-1 in the PVN. This increase in PIC expression was attenuated in Ang II+ETN rats. n=9-10 per group, *p<0.05 vs Control, #p<0.05 vs Ang II.

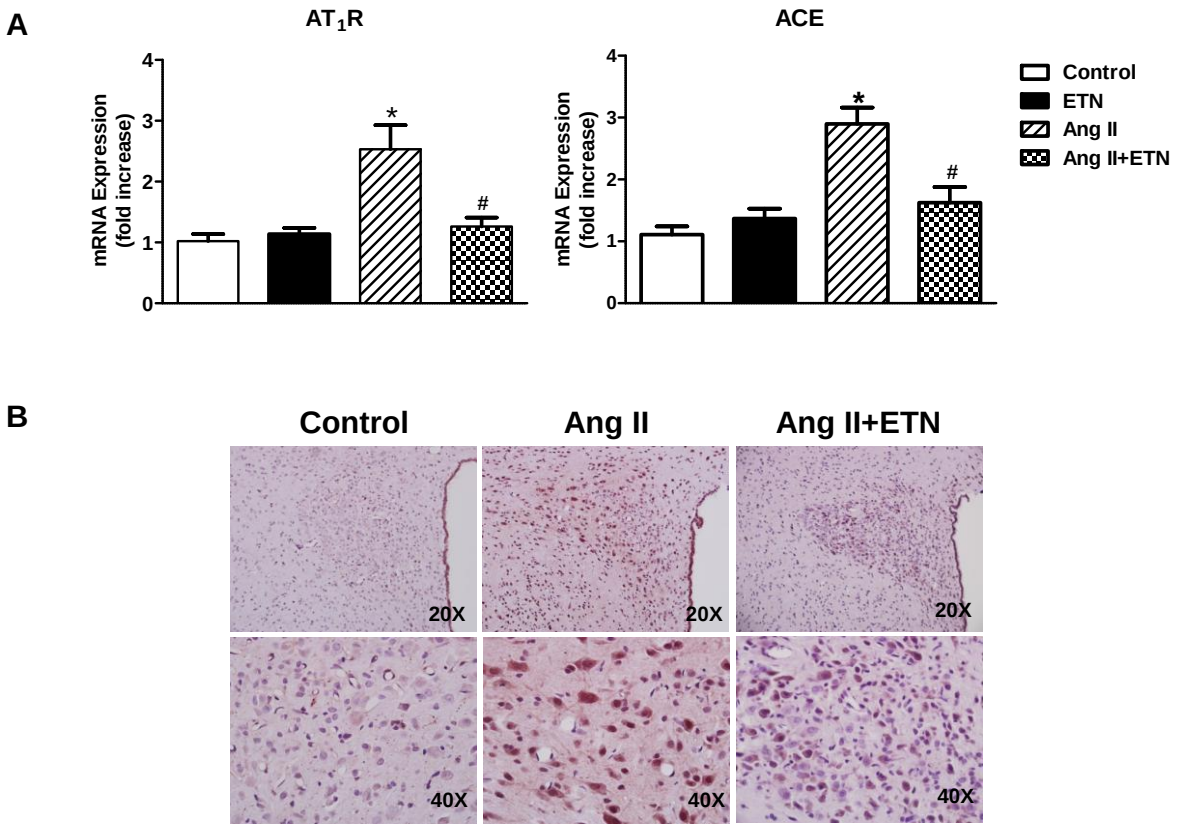

Figure 4.3. Effects of central TNF blockade pro-hypertensive RAS component expression in the PVN. Chronic, 28-day Ang II infusion increased the mRNA expression of AT_1R and ACE in the PVN, which was attenuated in Ang II+ETN rats (3A). Immunohistochemical staining against the AT_1R in the PVN followed a similar pattern of increase in Ang II-treated rats, but reduced following ICV etanercept administration (3B). n=9-10 per group, *p<0.05 vs Control, #p<0.05 vs Ang II.

Effect of Ang II Infusion on the Expression of RAS Components in the PVN. Both Ang II and TNF have been shown to modulate RAS component expression. To determine the manner by which Ang II infusion alters the expression of the pro- and anti-hypertensive components of the RAS in the PVN, we examined the mRNA expression levels of ACE, ACE2, AT_1R, AT_2R, and the Mas receptor by real time RT-PCR, and the protein expression of the AT_1R using immunohistochemistry. The gene expression of the pro-hypertensive RAS components (AT_1R and ACE) in the PVN were significantly increased in Ang II-infused rats when compared with

control rats; whereas in Ang II-infused rats treated ICV with ETN, these components were reduced (Figure 4.3A). Immunohistochemistry against AT_1R protein expression in the PVN showed a similar pattern of increase in Ang II-infused rats, but a reduction in ICV ETN-administered rats (Figure 4.3B). Conversely, the anti-hypertensive components of the RAS (ACE2, AT_2R and the Mas receptor) showed a decreased gene expression in Ang II-treated rats. These levels were increased in the Ang II+ETN group (Figure 4.4). This data suggests that in Ang II-induced hypertension, the pro- and anti-hypertensive components of the RAS are differentially regulated within the PVN in a deleterious manner. Additionally, ACE2 can increase during the normal RAS response. Here, in blocking TNF, which decreases ACE2 as well as the other anti-hypertensive RAS components, it possibly allows for

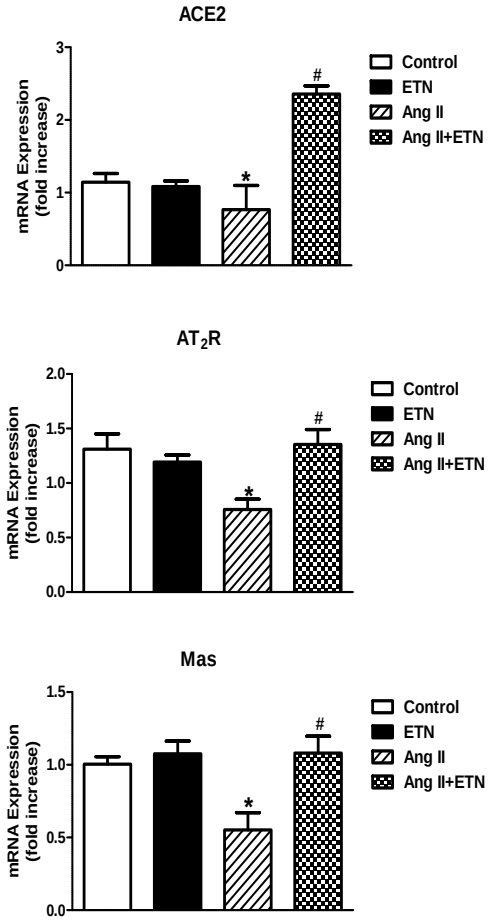

Figure 4.4. Effects of central TNF blockade anti-hypertensive RAS component expression in the PVN. Chronic, 28-day Ang II infusion decreased the mRNA expression of ACE2, AT_2R and the Mas receptor in the PVN. This decrease was reversed in Ang II+ETN rats. n=9-10 per group, *p<0.05 vs Control, #p<0.05 vs Ang II.

not only the recovery, but the overshoot observed in the ACE2 response by also not inhibiting the machinery (possibly including a ROS mechanism) responsible for modulating ACE2. This dysregulation is possibly through a PIC/TNF-mediated mechanism, and thereby perpetuating the Ang II-driven hypertensive response.

Effect of Ang II Infusion on the Expression of gp91*phox* and nNOS. Both Ang II and TNF have been shown to act through oxidative stress mediated pathways, especially within the PVN, in inducing elevated sympathetic outflow and a progressive hypertensive response. Therefore, we analyzed the PVN mRNA expression of gp91*phox*, the catalytic subunit of NADPH, and neuronal Nitric Oxide Synthase (nNOS; an indirect indicator of sympathoexcitation). Ang II infusion significantly increased the gp91*phox* subunit, and decreased nNOS mRNA expression, in Ang II infused rats; these changes were reversed in Ang II-infused rats treated with ETN (Figure 4.5). This data suggests that in Ang II-induced hypertension, oxidative stress (indicated by increased gp91*phox*) and sympathoexcitation (indicated by a decreased nNOS) increase, and that this is potentially through a TNF-driven mechanism.

DISCUSSION

The major findings in this study are as follows: 1) Chronic Ang II infusion increased mean arterial blood pressure and cardiac hypertrophy; 2) within the PVN, chronic Ang II infusion increased PICs such as TNF and IL-1β, as well as gp91*phox* and decreased nNOS; 3) Ang II infusion also led to an elevation in pro-hypertensive RAS component expression (ACE and AT_1R) and a decrease in anti-hypertensive RAS component expression (ACE2, AT_2R and Mas) within the PVN; 4) Ang II-infused rats treated ICV with etanercept, a soluble TNF receptor mimetic, prevented these changes, including attenuating MAP and PICs, and reversing the expression alterations observed between the pro- and anti-hypertensive RAS arms. These

findings demonstrate that the changes observed in Ang II-induced hypertension are regulated, at least in part, through the central actions of TNF and potentially via the dysregulation of components of the RAS within the hypothalamic PVN.

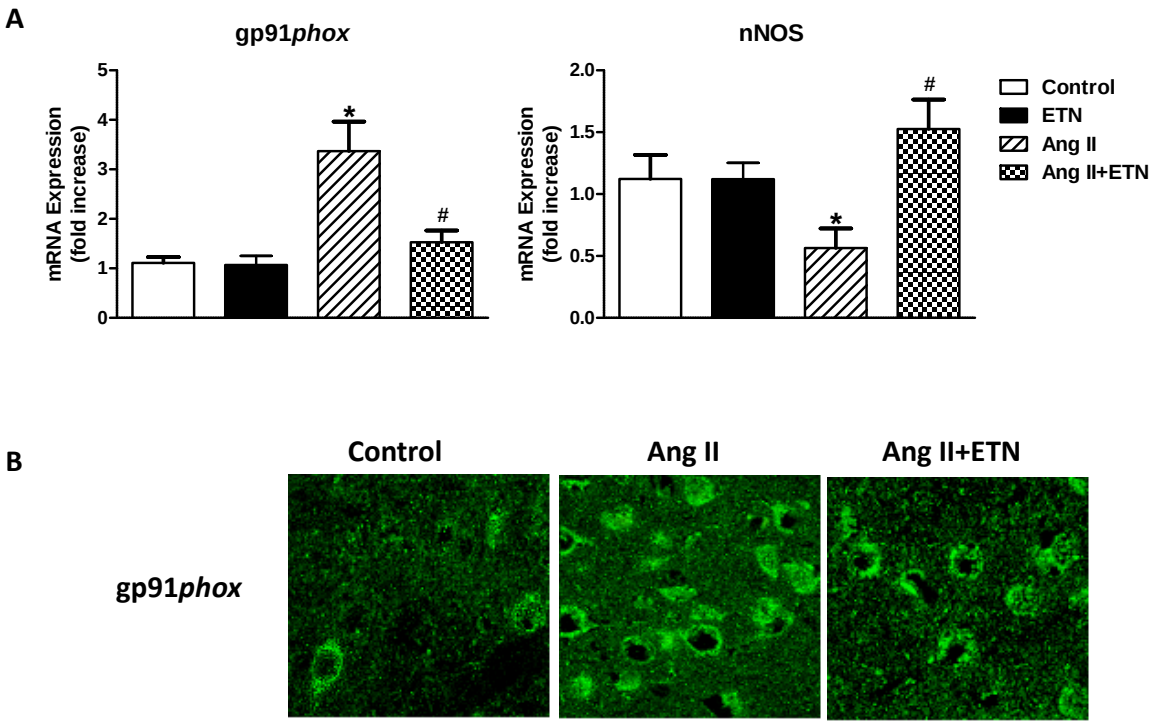

Figure 4.5. Effects of central TNF blockade on oxidative stress marker gp91*phox* and nNOS expression in the PVN. Ang II-infused rats demonstrated an increase in the mRNA expression of the NADPH oxidase catalytic subunit, gp91*phox*, while that of nNOS was decreased in the PVN (5A). These alterations were reversed in etanercept-treated rats. Similarly, immunofluorescence against gp91*phox* showed an increased neuronal expression in Ang II-infused rats, which was decreased in Ang II+ETN rats (5B). n=9-10 per group, **p*<0.05 vs Control, #*p*<0.05 vs Ang II.

Recent evidence suggests that hypertension is an inflammatory condition where various PICs such as TNF, IL-6 and IL-1β, both centrally and peripherally, have been shown to play an important role in the pathogenesis of hypertension (Chae, Lee et al. 2001; Veerasingham and

Raizada 2003; Sun, Li et al. 2004; Sriramula, Haque et al. 2008; Lu, Chen et al. 2009; Shi, Diez-Freire et al. 2010). A recent study from our lab demonstrated that chronic peripheral Ang II infusion results in increased production of PICs within the PVN (Kang, Ma et al. 2009). Blockade of TNF by etanercept has been shown to prevent renal damage in a genetic hypertensive rat model, as well as lower blood pressure in rats with Ang II- and salt-induced hypertension, suggesting a role for TNF in blood pressure regulation and renal injury (Muller, Shagdarsuren et al. 2002; Elmarakby, Quigley et al. 2006). Another study also showed that mice treated with etanercept had an attenuated hypertension and a blunted increase in superoxide production in response to Ang II (Guzik, Hoch et al. 2007). Etanercept is a soluble recombinant fusion protein the inhibits TNF by posing as a TNF receptor decoy and acting through competitive inhibition of TNF and an overall reduction in free TNF to act on endogenous receptors (Goffe and Cather 2003). Our present observations complement these findings and show that ICV administration of etanercept into the brain protects rats against Ang II-dependent cardiac hypertrophy and hypertension.

Ang II can act as a potent proinflammatory agent and stimulate the production of chemokines such as MCP-1, and PICs, such as TNF, IL-6 and IL-1β in the brain (Shi, Diez-Freire et al. 2010). TNF is commonly considered as one of the initiators of the pro-inflammatory cascade, which can induce production of other cytokines and chemokines (Zhang, Hein et al. 2006; Zhang, Xu et al. 2006). A recent study demonstrated that Ang II-induced hypertension involves activation of microglia and increased expression of PICs within the PVN (Shi, Diez-Freire et al. 2010). In our study, TNF blockade with etanercept decreased the expression not only of TNF, but of other cytokines such as IL-6, IL-1β, and chemokine MCP-1 within the PVN, supporting the hypothesis that PIC are involved in the Ang II-induced hypertensive response.

Excessive ROS production in brain cardio-regulatory centers such as the PVN can contribute to the neurogenic component of the hypertensive response by enhancing sympathetic activity and outflow (Paravicini and Touyz 2006). It has been shown that NADPH oxidase is the primary source of Ang II-induced ROS in neurons (Paravicini and Touyz 2006) and that treatment with the cell permeable superoxide dismutase (SOD) mimetic Tempol inhibits Ang II-mediated superoxide production and hypertension (Nishiyama, Fukui et al. 2001). Furthermore, TNF can induce activation of NADPH oxidase leading to enhanced oxidative stress and decreasing the bioavailability of NO (Zhang, Xu et al. 2006). Moreover, in the present study, nNOS was decreased in the PVN of Ang II-treated rats, but restored following ICV etanercept administration. Neuronal NOS is an inverse indirect indicator of sympathoexcitation, in that a decrease in nNOS correlates with an increase in sympathetic outflow. Therefore, a reduction in beneficial NO not only decreases with the reduction in nNOS expression and activity, but along with the rapid interconversion of NO to peroxynitrite ($ONOO^-$) with the ever increasing production of NADPH oxidase-derived superoxide. These factors combined can lead to an increased PIC response, sympathoexcitation and a continued propagation of neurgenic hypertension. The present study provides further support for these observations by showing that central TNF blockade with etanercept reduced the expression of gp91*phox* (the catalytic subunit of NADPH oxidase), restored nNOS, and ultimately decreased the changes associated with the hypertensive state.

In many animal models of hypertension, the expression of the AT_1R is upregulated in central cardiovascular regulatory centers, including the hypothalamic PVN (Veerasingham and Raizada 2003). In the brain, the AT_1R mediates the central effects of Ang II, including vasopressin release, water and salt intake and balance, and increased sympathetic drive, all of

which contribute to the development of hypertension (Paul, Poyan Mehr et al. 2006; von Bohlen und Halbach and Albrecht 2006). Both *in vitro* and *in vivo* studies have demonstrated the existence of a cross-talk between Ang II and TNF (Sasamura, Nakazato et al. 1997; Arenas, Xu et al. 2004), and we have also shown that attenuation of Ang II-induced hypertension in TNF knockout mice involves a decreased expression of AT_1R (Sriramula, Haque et al. 2008). Presently, we show that chronic Ang II infusion results in an increase in PVN ACE and AT_1R, both which were reduced following ICV etanercept infusion. Recently, the discovery of an alternate set of components of the RAS which may act as a counterbalance to the actions of the ACE/Ang II/AT_1R pathway added complexity to the understanding of RAS regulation, especially within the brain (Xu, Sriramula et al. 2011). These components, termed anti-hypertensive due to their cardio-protective effects, along with all the components of the pro-hypertensive RAS axis, are known to be expressed throughout the various central cardio-regulatory regions, including the PVN. In various experimental hypertensive models, these components (ACE2, AT_2R, Ang (1-7) and the Mas receptor) are shown to be downregulated, while the pro-hypertensive components are increased. This dysregulation may be the lynch pin trigger towards developing hypertension, but its management is poorly understood. In the present study, ICV treatment with etanercept resulted in reduction of Ang II-induced pro-hypertensive RAS component expression, including ACE and AT_1R upregulation in the PVN, as well as the restoration of the anti-hypertensive RAS components ACE2, AT_2R and the Mas receptor. As mentioned previously, ACE2 can increase during the normal RAS response, but following the eventual increase in TNF, it decreases ACE2 as well as the other anti-hypertensive RAS components. Thus, the TNF blockade possibly allows for not only the recovery, but the overshoot observed in the ACE2 response by not inhibiting the machinery responsible for

moderating and modulating ACE2. However, these same signaling mechanisms seem to have little to no effect on the AT_2R and Mas receptor gene expression increases. This/these mechanism(s) must be studied closely to delineate these observed differences. These results suggest that this RAS dysregulation and perpetuation of the hypertensive state may be the result of a pro-inflammatory response through the actions of TNF, but further studies are necessary to completely understand this mechanism.

In summary, chronic Ang II infusion resulted in cardiac hypertrophy and hypertension, and within the PVN, an increased PIC and gp91*phox* expression, and decreased expression of nNOS. Perhaps more importantly, Ang II-infused rats had an increased expression of the injurious pro-hypertensive RAS components ACE and AT_1R, and a decreased expression of the protective anti-hypertensive RAS components ACE2, AT_2R and the Mas receptor. Central blockade of TNF with etanercept resulted in attenuation of hypertension, cardiac hypertrophy and PIC expression, decreased oxidative stress, as well as a restored the balance between the protective and deleterious arms of the RAS, within the hypothalamic PVN. The beneficial effects of central TNF blockade in Ang II-induced hypertensive responses appears to be mediated by the returned balance of the central RAS components, especially within the PVN. It is important to note, however, that due to the administration of etanercept ICV, the TNF inhibitory effects may have impacted additional cardio-regulatory regions in the brain and elicited a similar response as in the PVN, but in light of its central integrative function versus the other regions, the PVN was of utmost concern. These other regions should also be investigated in future studies, as well as a closer look at the specific pathway between Ang II, TNF and the differential regulation of the RAS arms in the Ang II hypertension-induced animal model. Our findings provide further evidence and insight for the involvement of the RAS within the PVN and its interaction and

mediation through TNF in the neurogenic component of hypertension. Further exploration of these system interactions within the brain may be beneficial towards the development of novel hypertensive therapeutics.

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CHAPTER 5

NUCLEAR FACTOR-*kappa*B BLOCKADE IN THE PARAVENTRICULAR NUCLEUS MODULATES RENIN-ANGIOTENSIN SYSTEM COMPONENTS AND ATTENUATES HYPERTENSION

INTRODUCTION

Hypertension is a condition closely associated with the renin-angiotensin system (RAS) and increased expression of proinflammatory cytokines (PICs) and reactive oxygen species, in both systemic and local hypertensive responses (Chae, Lee et al. 2001; Zimmerman, Lazartigues et al. 2002; Davisson 2003; Gao, Wang et al. 2005; Ferrario and Strawn 2006; Granger 2006; Kang, Ma et al. 2009). Studies from our laboratory and others have shown that in hypertension, angiotensin II (AngII), PICs and ROS can increase the activity of the transcription factor Nuclear Factor-*kappa*B (NFκB), which in turn, can further increase PIC and ROS expression in a positive feed-forward manner (Allen, Zhuo et al. 2000; van den Berg, Haenen et al. 2001; Zhang, Wei et al. 2003; Bubici, Papa et al. 2006; Tian, Moore et al. 2007; Kang, Ma et al. 2009; Cardinale, Sriramula et al. 2010).

Within the brain, multiple cardio-regulatory regions exhibit a local RAS, including the hypothalamic paraventricular nucleus (PVN), which can synthesize and release both pro- and anti-hypertensive RAS component peptides (Davisson 2003; Xia and Lazartigues 2008). The PVN is recognized as a central integration site for the coordination of autonomic and neuroendocrine responses that regulate thirst, salt appetite and sympathetic outflow (Davisson, Oliverio et al. 2000; de Wardener 2001; Sriramula, Haque et al. 2008). AngII is a large peptide that cannot cross the blood-brain barrier (BBB). Therefore, it exerts its roles by acting on the circumventricular organs (CVOs), an area where the BBB is either weak or absent (Simpson 1981; Ganong 2000). Signals from these CVOs subsequently activate neurons within the various cardio-regulatory centers of the brainstem and hypothalamus, including the PVN (Ganong 2000), which can respond by locally producing components of the RAS and via sympathetic signals to the periphery (Kang, Ma et al. 2009).

Recent findings from our laboratory and others have shown that, with hypertension, PICs are increased in discrete brain sites, such as the PVN, and that signals from both the systemic and local RASs increase PICs and oxidative stress (Kang, Ma et al. 2009; Shi, Raizada et al.). Within the PVN, the RAS, PICs and ROS have been linked to increased sympathoexcitation and perpetuation of the hypertensive state (Sun, Li et al. 2004; Lu, Chen et al. 2009). Based upon the preceding evidence, we hypothesized that bilateral PVN blockade of NFκB would attenuate these observed regional changes which propagate the AngII-induced hypertensive response, including increases in PICs and ROS. To test this hypothesis, we blocked NFκB within the PVN using two different approaches: bilateral PVN NFκB decoy oligodeoxynucleotide infusion, or bilateral PVN microinjection of an Adenoviral vector containing a serine mutated Inhibitoy-*kappa*B (IκB) (AdIκB). These inhibition techniques block two separate locations in the NFκB transcription activation pathway, prior to entering the nucleus (AdIκB) and following nuclear translocation (NFκB decoy). Our results demonstrate that NFκB is integral to perpetuating the hypertensive state through transcriptional activation of the local RAS, PIC and ROS actions within the PVN, as well as acting as a tipping point modulator in the balance between the pro-hypertensive and the anti-hypertensive arms of the RAS. This data shows the important role that PVN specific NFκB plays in the neurogenic control of hypertension through RAS modulation.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (12 weeks old, 250-350grams, n=153) were used in this study. Animals were housed in a temperature- ($25 \pm 1^{o}C$) and light-controlled (12:12 hour light:dark cycle) room with free access to water and normal rat chow (0.4% salt). All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Louisiana State University in accordance with NIH guidelines.

Experimental Protocol. Rats were anaesthetized and implanted with radio-telemetry transmitters. Following a 7-day recovery, bilateral cannulae were placed into the PVN. Fourteen-day osmotic minipumps (Alzet, model 2002) with an infusion rate of 0.5µl/h were filled with AngII (Bachem, 200ng/kg/min) dissolved in 0.9% saline, or saline alone, and implanted subcutaneously in the retroscapular area. Simultaneously, osmotic minipumps (infusion rate of 0.11µl/h; Alzet, model 1004) were filled with NFκB decoy or control scrambled decoy oligodeoxynucleotide (2ng/kg/min; Sigma), dissolved in aCSF, implanted subcutaneously in the retroscapular area and connected to the cannula. The NFκB decoy concentration was determined from a previous pilot study in rats using three different doses, 200pg/kg/min, 2ng/kg/min and 200ng/kg/min. The 2ng/kg/min dose was found to be optimal, while the highest dose caused increased mortality and the lowest dose did not produce complete NFκB inhibition as measured using an NFκB (p65) activity assay. Rats were divided into 4 groups: 1) No treatment (Controls; n=11); 2) Saline minipump + bilateral PVN NFkB decoy (Saline+NFκB decoy; n=8); 3) AngII minipump + bilateral PVN scrambled decoy (AngII+Scramble decoy; n=8); and 4) AngII minipump + bilateral PVN NFκB decoy (AngII+NFκB decoy; n=20). Another group of rats were also implanted with radio-telemetry transmitters and allowed a 7-day recovery. These rats were injected ($2x10^{10}$ pfu/ml, 100nL) bilaterally intra-PVN with an Adenoviral vector (Ad) containing IκB serine mutated at the S23A/S36A positions (AdIκB), or a control Ad with an empty cassette region (AdEmpty; both adenoviruses obtained from Gene Transfer Vector Core, University of Iowa, Carver College of Medicine) using a 1µl Hamilton syringe, as previously described (Feng, Yue et al. 2008). Also in this group of rats, 14-day osmotic minipumps (Alzet, model 2002; 0.5µl/h) were filled with AngII dissolved in 0.9% saline or saline alone and implanted subcutaneously into the retroscapular area. These rats were also divided into 4 groups: 1) No

treatment (Controls; n=10); 2) Saline minipump + bilateral PVN AdIκB (Saline+AdIκB; n=9); 3) AngII minipump + bilateral PVN AdEmpty (AngII+AdEmpty; n=8); and 4) AngII minipump + bilateral PVN AdIκB (AngII+AdIκB; n=21). A final group of rats were treated with AngII alone (n=15) and used for western blot, immunohistochemical and electron paramagnetic resonance (EPR) analysis. All rats were euthanized using a high ketamine+xylazine dose after 14 days of blood pressure recordings and brain tissue was collected for mRNA and protein analysis (1.8μg mRNA and 8-12μg protein extracted/side). All rats that received treatment unilaterally into the PVN or had malfunctioning pumps (based upon post-mortem analysis) were excluded from the final analysis (success rate: bilateral cannulation ~ 78%; bilateral microinjection ~ 65%). Chow salt (Na^+) content did not appear to have any effect on pressure response. A $p<0.05$ was considered significant.

Blood Pressure Measurement. Blood pressure was measured continuously in conscious rats implanted with radio-telemetry transmitters (Model TA11PA-C40, Data Sciences International, St. Paul, MN). Rats were anesthetized with a ketamine (90 mg/kg) and xylazine (10 mg/kg) mixture (i.p.) and placed dorsally on a heated surgical table. An incision was made on the ventral surface of the left leg, and the femoral artery and vein were exposed and bluntly dissected apart. The femoral artery was ligated distally, and a small clamp was used to temporarily interrupt the blood flow. The catheter tip was introduced through a small incision in the femoral artery, advanced ~6 cm into the abdominal aorta such that the tip was distal to the origin of the renal arteries, and secured into place. The body of the transmitter was placed in to the abdominal cavity and sutured to the abdominal wall. The abdominal musculature was sutured and the skin layer was closed. Rats received benzathine penicillin (30000 U, i.m.) and buprenorphine (0.1

mg/kg, s.c.) immediately following surgery and 12 h postoperatively. The rats were allowed 7-day surgical recovery.

Bilateral Cannula Implantation or Intra-PVN Injections. Rats to receive NFκB decoy or scrambled oligodeoxynucleotide decoy were anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg) mixture (i.p.). The rats were placed in a stereotaxic instrument (Kopf instruments; Tujunga, CA) and the skull was exposed through an incision on the midline of the scalp. After bregma was identified, the coordinates for the PVN were determined from the Paxinos and Watson (2007) rat atlas, at 1.8 mm posterior and 7.9 mm ventral to the zero level. Rats were implanted with custom designed bilateral cannulae (Plastics One; Roanoke, VA) and subsequently attached to osmotic mimipumps via sterile vinyl tubing. For adenovirus microinjections, we followed the same surgical procedure except that rats were injected bilaterally intra-PVN with AdIκB or AdEmpty virus using a 1μl Hamilton syringe with a custom made needle/tip and injector plunger that advances flush with the needle tip (Hamilton Company USA) at ±0.4 mm lateral to the bregma. ICV cannulated rats were examined for cannula placement during cryostat slicing with crystal violet staining prior to PVN punching and immunohistochemical slicing (bilateral cannulation ~ 78%; n=22). Microinjection locations were also determined via crystal violet staining prior to PVN punching and immunohistochemical slicing (bilateral microinjection ~ 65%; n=24). At this point, animals identified as receiving unilateral or no treatments were removed from the final analysis. Animals not able to be examined via staining methods were assessed based on pressure recordings, as the unilaterally-treated rats had a distinctive blood pressure range from the AngII+Scramble decoy and AngII+AdEmpty and the AngII+ NFκB decoy and AngII+ AdIκB treatment groups.

Detection of Total ROS, Superoxide and Peroxynitrite in PVN Tissue. One of the most

sensitive and definitive methods of total ROS, superoxide and peroxynitrite production is electron spin resonance (EPR). In this study, we utilized an established technique for ROS detection in tissue using EPR and spin traps as previously described (Cardinale, Sriramula et al. 2010; Guggilam, Cardinale et al. 2011).

RNA Isolation and Real-Time RT-PCR . Total RNA was extracted from the PVN using TRI reagent (Invitrogen), and cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad) as previously described (Guggilam, Haque et al. 2007; Sriramula, Haque et al. 2008). The mRNA expression levels of TNF, IL-1β, IL-6, MCP-1, AT_1R, ACE, ACE2, the Mas receptor and nNOS were determined using previously published specific custom made primers (Guggilam, Haque et al. 2007; Sriramula, Haque et al. 2008; Agarwal, Haque et al. 2009; Elks, Mariappan et al. 2009; Mariappan, Elks et al. 2009). GAPDH was used as the housekeeping gene. Real-time RT-PCR (qRT-PCR) was performed in 384 well PCR plates using Bio-Rad PCR Master Mix (The iTaq SYBR™ Green Supermix with ROX) and the ABI Prism 7900 sequence detection system (Applied Biosystems). The PCR cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles (15 s at 95°C, 1 min, at 60°C). Finally, a dissociation step (15 s at 95°C, 15 s, at 60°C and 15 s at 95°C) was added to check the melting temperature of the specific PCR product.

Western Blot. Western blot analysis was performed according to standard protocols. The PVN tissue was homogenized with RIPA lysis buffer. Equal amounts of protein (5µg) were SDS-PAGE separated on 10% wt/vol gels, transferred to PVDF membrane (Immobilon-P, Millipore) and blocked with 1% BSA in TBS-T at room temperature for 60 min. The membranes were subjected to immunoblot analyses with anti-AT_1 (Abcam), anti-MasR (Santa Cruz) and anti-GAPDH (Santa Cruz) antibodies (1:200 dilution). Immunodetection was accomplished with a

horse radish anti-rabbit or anti-goat secondary antibody (1:2000 dilution) using an enhanced chemiluminescence kit (Amersham) as previously described (Sriramula, Haque et al. 2008). The data were quantified using ImageJ software and were normalized to GAPDH expression.

Immunohistochemical Analysis. Rats (n=4 in each group) were transcardially perfused with 200ml of ice-cold PBS (7.4 pH; 0.1M) followed immediately by 200 ml of 4% paraformaldehyde in PBS. The brains were removed, postfixed in 4% paraformaldehyde solution for 2 hours, and transferred to a phosphate buffer containing 20% sucrose (pH 7.4) and stored overnight. For immunostaining, 10µm coronal sections from paraffin embedded brains were collected on slides. First the sections were incubated with 0.3% H_2O_2 in methanol for 10 minutes. For antigen retrieval, citrate buffer with microwave heating technique is used. Then the sections were incubated with 1.5% goat or rabbit serum in PBS containing 0.3% Triton X100 for 30 minutes. The sections were incubated with primary antibodies (TNF, 1:100 dilution, anti-goat; Santa Cruz; nNOS, 1:100 dilution, anti-goat; Santa Cruz) overnight at 4°C followed by incubation with biotinylated goat-anti rabbit or rabbit-anti goat secondary antibodies for 60 minutes, and stained with Vectastain ABC kit (Vector Laboratories) according to the manufacturer's instructions. Each step was followed by washing the sections with PBS containing 0.3% Triton X100. Sections incubated without primary antibody were used as negative controls.

Determination of IκB Overexpression by Immunofluorescence. For detection of IκB in PVN tissue, slides were incubated overnight at 4ºC with a 1:100 dilution of goat polyclonal anti-IκB (Santa Cruz) as previously described (Cardinale, Sriramula et al. 2010).

p65 Activity Assay for Assessment of NF-kB Binding Activity. The NFκB/p65 TransAM Active ELISA kit (Active Motif) was used to measure the binding activity of free NFκB p65 in nuclear extracts (Active Motif). The extraction and analysis were done using a sandwich ELISA

method and in accordance to the manufacturer's instructions. Procedure yields 0.15-0.25mg of nuclear extract from PVN tissue at 3-5mg/ml. NFκB/p65 Active ELISA detection levels for nuclear extracts between 0.5-1μg/well at 450nm.

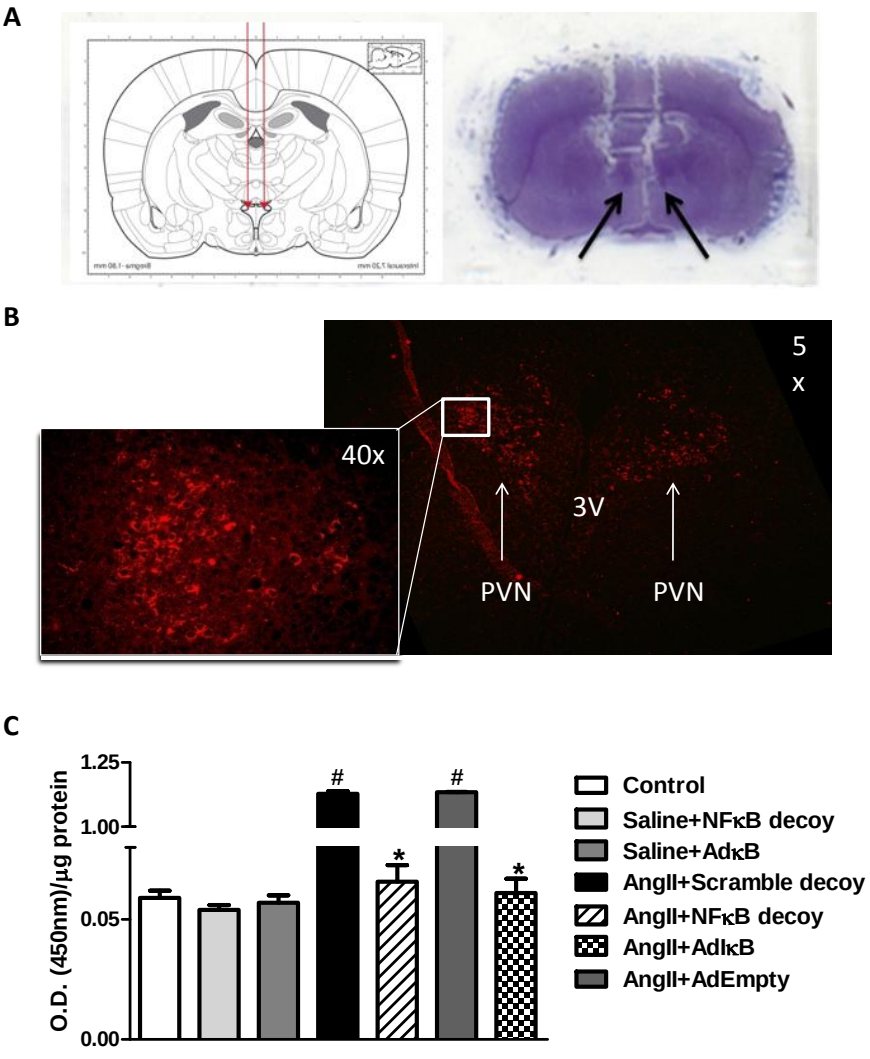

Figure 5.1. PVN specific inhibition of NFκB. (**A**) Schematic showing cannulae placement/microinjection stereotaxic location coordinates. (**B**) Representative immunofluorescence image for IκB is localized in the PVN following AdIκB bilateral microinjection into control rat. (**C**) NFκB p65 activity assay showing increased activity in the PVN of the AngII-treated groups when compared to control groups. p65 activity is decreased in AngII-treated rats following bilateral PVN NFκB decoy infusion or AdIκB microinjection. n=5-6 rats per group, *p<0.05 vs respective AngII-treated rats, $^{\#}p$<0.05 vs respective control-treated rats.

Statistical Analysis. All results are expressed as mean±SEM. For statistical analysis of the data, student's *t* test, one-way ANOVA or repeated measures ANOVA followed by Bonferroni's correction *post hoc* was performed (GraphPad Prism v5.0 for Windows, GraphPad Software, San Diego California, USA) to determine differences among groups. A value of $p<0.05$ was considered statistically significant.

RESULTS

NFκB Blockade in the PVN Reduces p65 Subunit Binding Activity. To determine the role of NFκB inhibition in the central regulation of blood pressure, we either bilaterally infused NFκB decoy oligodeoxynucleotide into the PVN via a fixed cannula (Figure 5.1A) or bilaterally microinjected an Adenovirus encoding serine mutated IκB to overexpress IκB and inhibit NFκB within the PVN. Figure 5.1B shows the localization of IκB gene overexpression specifically within the PVN following bilateral PVN AdIκB

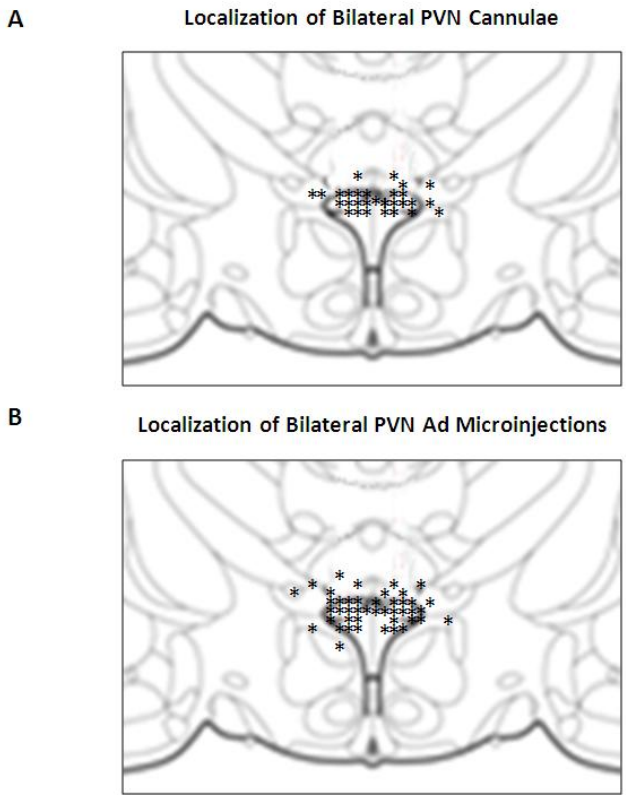

Figure 5.2. Localizaion of treatment sites within the PVN. Bilateral cannulae placement (**A**; n=22) and microinjection (**B**; n=24) sites as determined by post-mortem analysis of the brain using crystal violet staining prior to punching the PVN and immunohistochemical slicing. Rats demonstrating unilateral treatments also demonstrated an elevated MAP from the bilaterally treated AngII+Scramble decoy, AngII+AdEmpty, AngII+ NFκB decoy and AngII+ AdIκB groups.

microinjection, as indicated by enhanced fluorescence protein expression. Localization of injection sites of examined rats are schematically represented in Figure 5.2. To determine the efficacy of the two methods inhibiting NFκB activity, an NFκB p65 subunit activity assay was conducted following 14-day treatment in the PVN. The p65 subunit activity was dramatically increased in the PVN in the two AngII-treated groups versus their respective controls (Figure 5.1C). This increase in activity was attenuated in both AngII+NFκB decoy and Ang II+AdIκB-treated rats. This data indicates that NFκB is increased within the PVN during the AngII-induced hypertensive response and that the use of NFκB decoy or AdIκB can potently, and site specifically, inhibit the activity of NFκB.

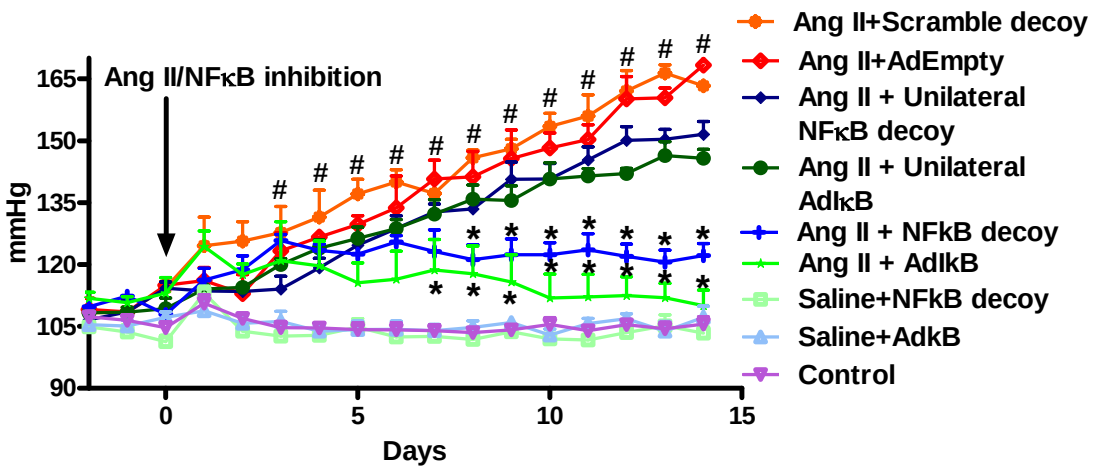

Figure 5.3. Effects of NFκB inhibition on AngII-induced increases in MAP. AngII continuously increased MAP in centrally treated control rats. Bilateral NFκB decoy infusion or AdIκB microinjection into the PVN decreased this AngII-induced increase in MAP. Unilateral PVN NFκB blockade only partially decreased the AngII-induced MAP. n=7-8/group, *p<0.05 vs respective AngII-treated rats, $^{\#}p$<0.05 vs respective control-treated rats.

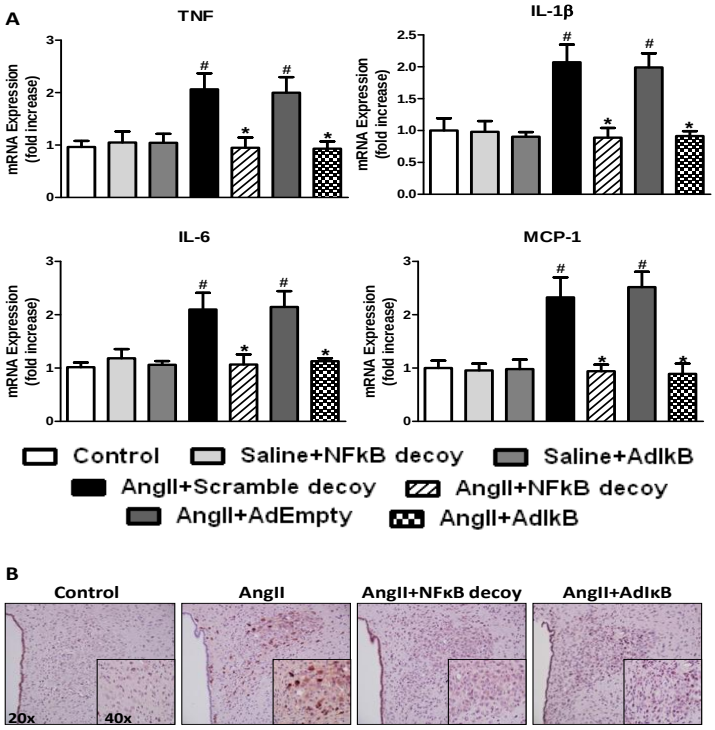

Figure 5.4. Effects of NFκB inhibition on AngII-induced increases in PIC expression. (**A**) mRNA expression of the PICs TNF, IL-1β, IL-6 and MCP-1 in the PVN of Ang II-infused groups are increased versus control groups. Expression is decreased following bilateral PVN NFκB decoy infusion or AdIκB microinjection. (**B**) Immunohistochemical staining for TNF is increased in the PVN in AngII-infused rats (AngII alone) versus Controls (no treatment). Staining was decreased in rats treated bilaterally into the PVN via NFκB decoy infusion or AdIκB microinjection. Images shown represent results observed in preparations from 4 to 6 rats. n=6-8/group, *p<0.05 vs respective AngII-treated rats, $^{\#}p$<0.05 vs respective control-treated rats.

NFκB Blockade in the PVN Attenuates the AngII-Induced Blood Pressure Response. To assess the effect of NFκB inhibition in the PVN on AngII-induced hypertensive response, mean arterial pressure (MAP) was measured using a radio-telemetry system (Figure 5.3). Chronic 14-day AngII infusion significantly increased the MAP in rats that received scramble decoy or AdEmpty treatment versus their respective saline-infused controls (Saline+NFκB decoy and Saline+AdIκB). In contrast, the MAP in AngII+NFκB decoy and AngII+AdIκB had a significantly reduced MAP, though the MAP was not decreased to control levels. Rats receiving either treatment unilaterally, though showing a reduced MAP versus the AngII control groups

was not reduced as effectively as bilateral treatments. This data indicates that NFκB within the PVN plays a contributing role to blood pressure response in AngII-induced hypertension.

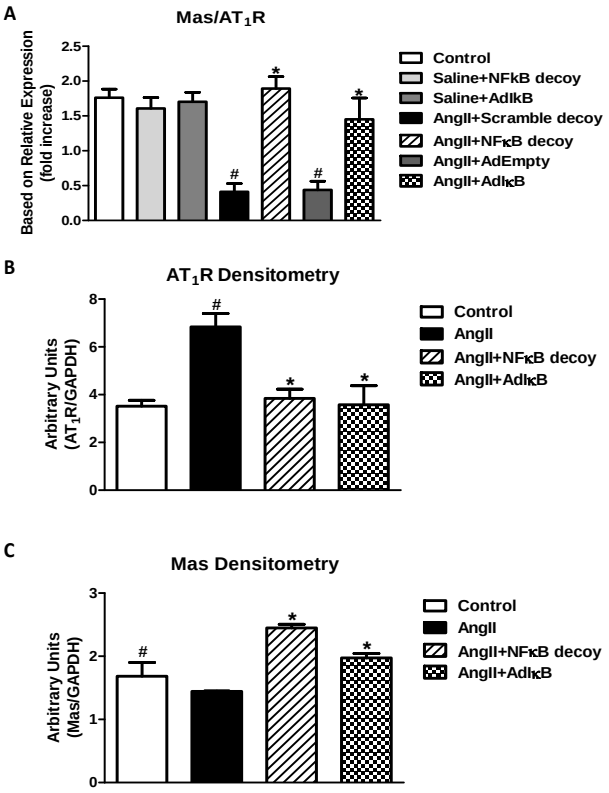

Figure 5.5. Effects of NFκB inhibition on AngII-induced changes in RAS component expression. (**A**) Mas/AT_1R ratio and western densitometry for AT_1R (**B**) and the Mas receptor (**C**). Bilateral PVN NFκB decoy infusion or AdIκB microinjection decreased the AngII-induced increase in AT_1R and increased the AngII-induced decrease in Mas receptor protein expression. n=6-8 in each group, $^{*}p<0.05$ vs respective AngII-treated rats, $^{\#}p<0.05$ vs respective control-treated rats.

NFκB Blockade Decreases PIC Expression in the PVN. To study the effect of NFκB blockade on PIC expression in the PVN, expression levels were measured by real-time PCR. AngII infusion significantly increased the mRNA expression of TNF-α, IL-1β, IL-6, and the chemokine MCP-1 in the PVN versus control rats. However, bilateral PVN NFκB decoy infusion or bilateral microinjection of AdIκB into the PVN attenuated these changes in PIC gene expression (Figure 5.4A). Furthermore, immunohistochemistry against TNF showed an increase in staining in AngII-treated rats (AngII alone) versus Controls (no treatment) (Figure 5.4B). This protein expression was reduced via bilateral PVN NFκB decoy infusion or bilateral AdIκB microinjection into the PVN, demonstrating that through NFκB inhibition within the PVN, PIC levels are reduced within this hypothalamic region.

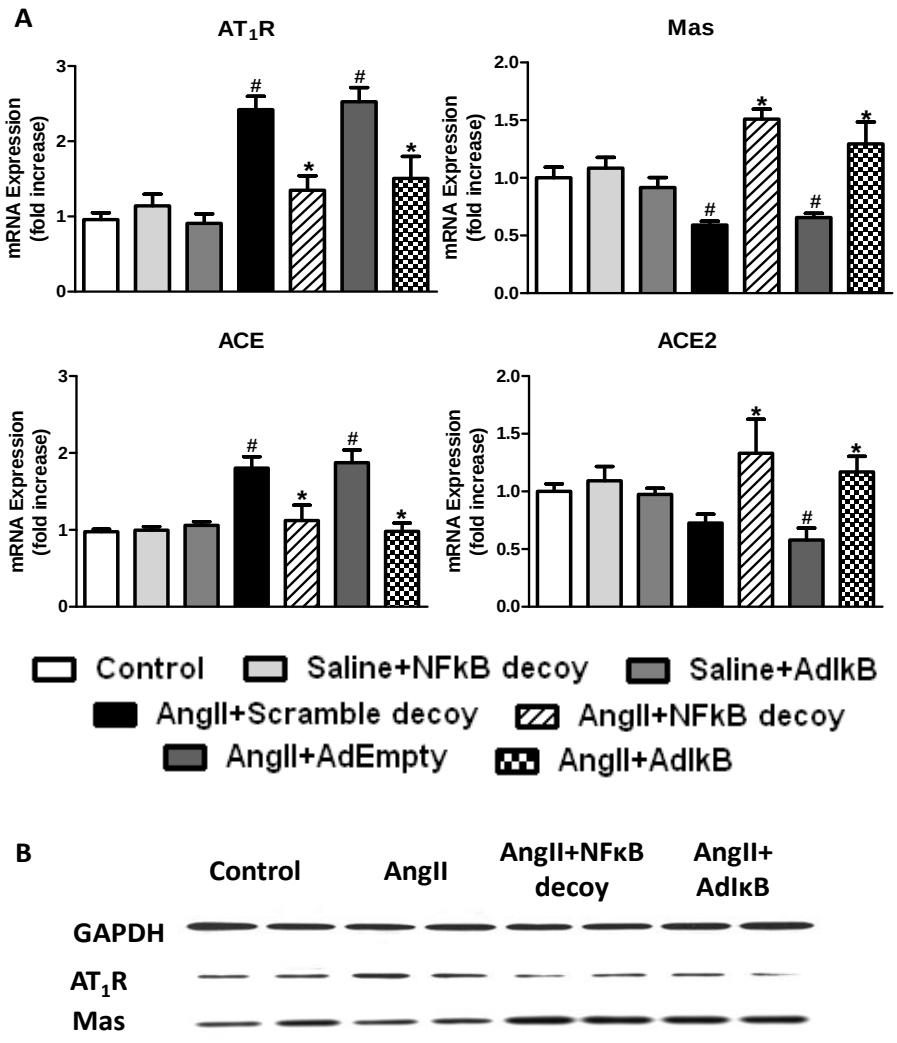

Figure 5.6. Effects of NFκB inhibition on Ang II-induced changes in RAS component expression. (**A**) mRNA expression of AT_1R and ACE was increased, while ACE2 and the MasR were decreased in the Ang II-treated rat groups when compared to controls. Bilateral PVN NFκB decoy infusion or AdIκB microinjection reversed these changes. (**B**) Western blots showed that AT_1R is increased, while the MasR is decreased in Ang II-treated rats (Ang II alone) when compared to Controls (no treatment). Bilateral PVN NFκB decoy infusion or AdIκB microinjection reversed these changes. n=6-8/group, $^{*}p<0.05$ vs respective Ang II-treated rats, $^{\#}p<0.05$ vs respective control-treated rats.

NFκB Blockade Effects RAS Component Expression in the PVN. To study the effect of NFκB on RAS components, the expression levels of ACE and ACE2 by PCR, and AT_1R and

Mas receptor (MasR) by PCR and western blot, were analyzed. AngII infusion significantly increased the mRNA expression of the AT_1R and ACE in the PVN when compared with control rat groups, and decreased the ACE2 and MasR expression versus their respective control groups (Figure 5.6A). Bilateral NFκB decoy infusion or microinjection of AdIκB into the PVN reversed these gene expression changes. AngII also decreased the Mas/AT_1R ratio within the PVN, which was reversed in AngII+NFκB decoy and AngII+AdIκB groups (Figure 5.5A). These results were further confirmed for AT_1R and MasR protein levels in the PVN by western blot (Figure 5.6B) and densitometric (Figure 5.5B and C) analysis. These results show that NFκB plays a modulatory role in RAS component expression in the PVN during the AngII-induced hypertensive response.

NFκB Blockade Reduces ROS Production in the PVN. To study the effects of NFκB blockade on ROS production in the PVN, ROS levels were measured by EPR analysis (Figure 5.7).

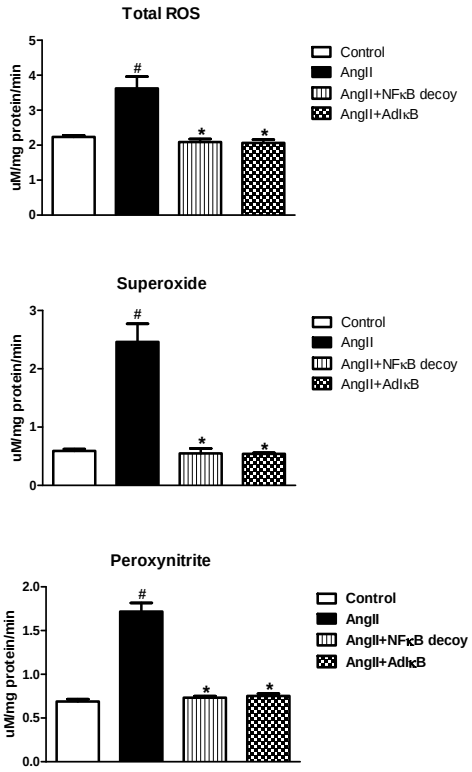

Figure 5.7. Effects of NFκB inhibition on Ang II-induced changes in ROS levels. AngII-infused rats (Ang II alone) had an increase in total ROS, superoxide and peroxynitrite within the PVN versus Control rats (no treatment). Bilateral PVN NFκB decoy infusion or AdIκB microinjection decreased these changes in ROS within the PVN. EPR spectra (right) for total ROS and superoxide. Graphical data when compared to total protein/sample. n=5-6/group, *$p<0.05$ vs respective Ang II-treated rats, $^{\#}p<0.05$ vs respective control-treated rats.

Total ROS, superoxide and peroxynitrite levels were significantly increased in the PVN of AngII-treated rats (AngII alone) versus Controls (no treatment). These ROS levels were decreased to normal levels following chronic bilateral NFκB decoy infusion or AdIκB microinjection into the PVN, indicating the role that NFκB plays in increasing ROS during the hypertensive response.

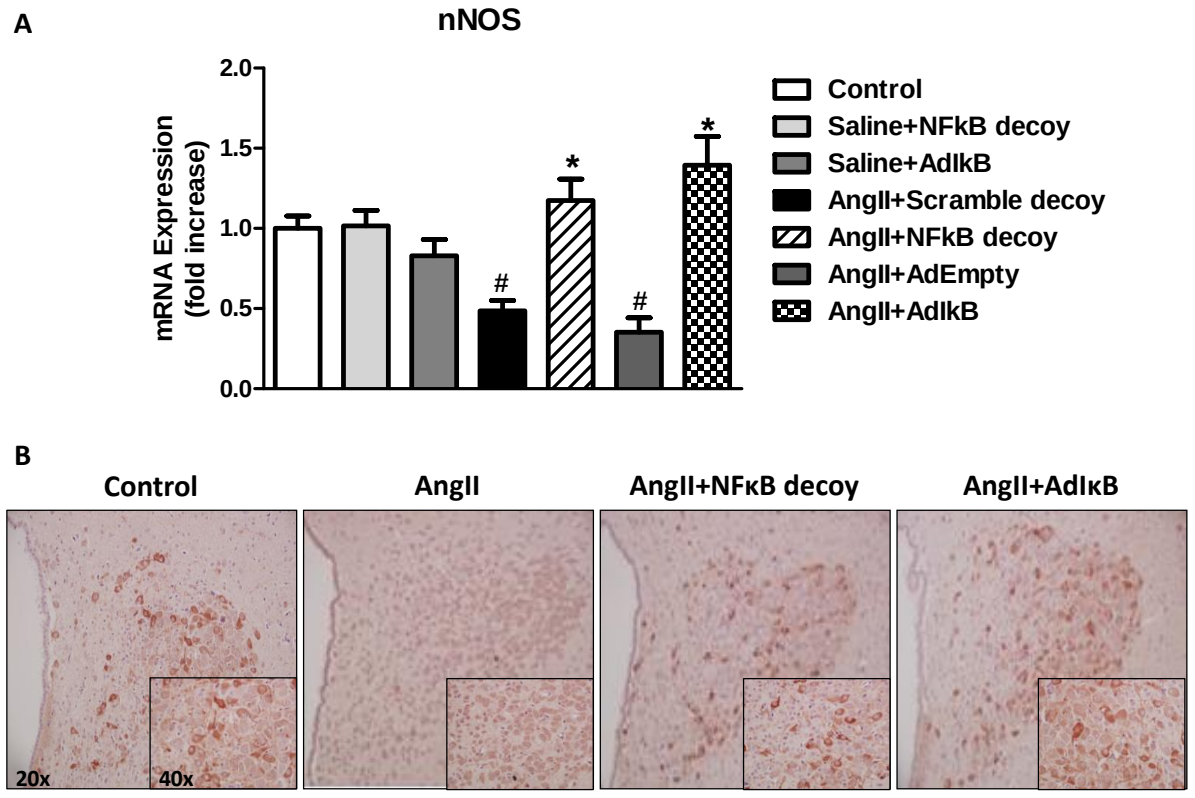

Figure 5.8. Effects of NFκB inhibition on Ang II-induced increases in nNOS expression. (**A**) mRNA expression of nNOS in the PVN of Ang II-infused rat groups is decreased versus control rat groups. Expression is increased following bilateral PVN NFκB decoy infusion or AdIκB microinjection. (**B**) Immunohistochemical staining for nNOS is increased in the PVN in AngII-infused rats (Ang II alone) versus Controls (no treatment). Staining was increased in rats treated bilaterally into the PVN via NFκB decoy infusion or AdIκB microinjection. Images shown represent results observed in preparations from 4 to 6 rats. n=6-8/group, *p<0.05 vs respective Ang II-treated rats, $^{\#}p$<0.05 vs respective control-treated rats.

NFκB Blockade Increases nNOS Expression in the PVN. Neuronal NOS (nNOS) expression is an indirect indicator of sympathoexcitation. In AngII-treated rat groups, nNOS mRNA was significantly decreased when compared to the control rat groups (Figure 5.8A). Bilateral NFκB decoy infusion or AdIκB microinjection into the PVN reversed and elevated these AngII-induced changes even beyond that of the normotesive controls. These results were further confirmed with immunohistochemistry against nNOS (Figure 5.8B), where AngII-infused rats (AngII alone) had decreased nNOS presence versus Controls (no treatment). Expression levels were elevated in AngII+NFκB decoy and AngII+AdIκB groups, indicating that through reduction of NFκB, nNOS, and potentially sympathoexcitation, were beneficially decreased in the AngII-induced hypertensive state.

DISCUSSION

In the present study, we investigated the effects of site specific bilateral hypothalamic PVN blockade of NFκB on the AngII-induced hypertensive response. The salient findings of this study are as follows: 1) Peripheral AngII infusion increases MAP and bilateral PVN NFκB blockade attenuates this elevated MAP; 2) Peripheral AngII infusion, within the PVN, increases PICs, ROS and pro-hypertensive (ACE and AT_1R) RAS components, while decreasing the anti-hypertensive (ACE2 and Mas) RAS components; 3) bilateral PVN specific NFκB inhibition not only decreases PVN PICs, but also modulates RAS component expression, such as decreasing ACE and AT_1R expression and increasing ACE2 and MasR expression; 4) PVN blockade of NFκB leads to an increase in nNOS expression and a decrease in $O_2^{\bullet -}$ and peroxynitrite ($OONO^{\bullet}$) levels. These results indicate that the NFκB-modulated inflammatory response acts as a double-edged sword that not only increases the deleterious pro-hypertensive arm of the RAS, but also decreases the protective anti-hypertensive arm of the RAS, possibly through a ROS mechanism.

The NFκB response is triggered by phosphorylation of IκB, which then becomes ubiquinated, is degraded, and frees NFκB, allowing its nuclear translocation to act on κB binding sites and commence transcription (Barnes and Karin 1997). In this study, two approaches were utilized to block NFκB within the PVN. AdIκB binds to NFκB similar to endogenous IκB, but serine mutations at the S32A/S36A positions prevent the IκB phosphorylation and ensuing NFκB release, thwarting its capability to translocate into the nucleus and transcribe target genes (Gilmore 2006). Decoy oligodeoxynucleotides act by targeting and adhering to the *cis*-element binding sites of free NFκB, thereby preventing its binding to κB gene binding sites and also blocking subsequent gene transcription (Morishita, Sugimoto et al. 1997; Morishita, Higaki et al. 1998). These two methods block NFκB transcription capabilities at two separate activation pathway locations: prior to entering the nucleus (AdIκB), and after being freed from IκB (NFκB decoy). This redundancy of blockade effectively demonstrates the role of PVN NFκB in AngII-induced hypertension, potentially signifying that there are no tangential pathways activated in the interim between NFκB release from IκB and NFκB's nuclear translocation. Previous findings from our lab investigating the role of the PVN in AngII-induced hypertension demonstrated that NFκB within the PVN was differentially regulated along with PIC and oxidative stress genes and proteins (Kang, Ma et al. 2009). The study presented the involvement of brain NFκB in regulating the hypertensive response, a previously novel proposal. However, in the study, intracerebroventricular (ICV) infusion of PDTC, a known antioxidant (Moellering, McAndrew et al. 1999), was used to investigate NFκB in the PVN in hypertension, indicating that NFκB could have been reduced through an antioxidant mechanism rather than direct NFκB blockade. Also, due to ICV administration, it could not rule out the possible involvement of other affected cardio-regulatory regions for the observed reduction in the hypertensive response. Therefore, the

exact role and proposed involvement/mechanism of NFκB within the PVN was still uncertain. The current study looks specifically at NFκB blockade within the PVN in the AngII-induced hypertensive response, delineates the involvement of PVN NFκB in regulating ROS and PIC expression, and potentially shows that NFκB serves as a tipping point trigger between the pro-hypertensive and protective anti-hypertensive arms of the RAS within the PVN.

Extensive evidence has implicated the RAS in the brain in hypertension (Gorbea-Oppliger and Fink 1995; Davisson 2003; Veerasingham and Raizada 2003). Within the PVN, an important central cardio-regulatory region, AngII is increased in multiple hypertensive animal models and PVN blockade of the AT_1R partially inhibits the effects of AngII-induced hypertension (Gorbea-Oppliger and Fink 1995; de Wardener 2001; Veerasingham and Raizada 2003; Silva, Santos et al. 2005; Kang, Ma et al. 2009). In the current study, following NFκB blockade within the PVN, ACE and the AT_1R were decreased, thereby limiting the effect that AngII could have on perpetuating AngII-induced hypertension. Moreover, and perhaps more importantly, NFκB inhibition also increased ACE2 and MasR expression, as well as improved the Mas/AT_1R ratio, indicating enhancement of the protective anti-hypertensive axis of the RAS. ACE2 is the counterpoint to ACE, in that instead of converting AngI to AngII, AngI and AngII are converted to Ang(1-7) (Xu, Sriramula et al.), which acts on the MasR to elicit actions opposing those of AngII, including vasorelaxation and decreased sympathetic activity (Diez-Freire, Vazquez et al. 2006; Ferrario 2006; Yamazato, Yamazato et al. 2007). Our results agree with other findings detailing the anti-hypertensive actions of ACE2 and the MasR (Diez-Freire, Vazquez et al. 2006; Yamazato, Yamazato et al. 2007), including their interplay with, and decreased activity following, AT_1R activation within the brain (Ferrario 2006; Feng, Yue et al. 2008; Xia, Feng et al. 2009; Feng, Xia et al. 2010). Combined, these results suggest NFκB as an

important tipping point between the protective (ACE2/Ang(1-7)/Mas) and non-protective (ACE/AngII/AT_1R) arms of the RAS, and that NFκB blockade promotes the more beneficial actions of ACE2 and the MasR.

The association between the RAS and elevated PICs in hypertension has often been explored, with many confirming more than a casual relationship between these two systems in the disease state (Chae, Lee et al. 2001; Sun, Li et al. 2004; Sriramula, Haque et al. 2008; Lu, Chen et al. 2009). NFκB is a key regulator of the PIC expression and inflammatory response speculated to have a role in the inflammatory component of hypertension (Cowling, Gurantz et al. 2002; Kang, Ma et al. 2009; Cardinale, Sriramula et al. 2010). Here, we show that bilateral NFκB inhibition in the PVN reduces PIC expression, establishing not only the definitive involvement of NFκB within the PVN in regulating the AngII-induced hypertensive response, but also that PICs may play a role in RAS modulation. Recently, ROS, especially $O_2^{\bullet-}$, have been shown as important signaling factors within the brain for enhancement of sympathetic outflow and increased hypertensive response through both AngII and PIC mechanisms (van den Berg, Haenen et al. 2001; Zhang, Wei et al. 2003; Zimmerman, Lazartigues et al. 2004; Guggilam, Haque et al. 2007). In this study, bilateral NFκB blockade reduced the ROS response, including that of $O_2^{\bullet-}$, thus potentially inhibiting one of the mechanistic pathways by which sympathoexcitation is modulated. These results are further confirmed by previous experiments in our lab showing a reduction of renal sympathetic nerve activity in AngII-treated rats following ICV administration of the ROS scavenger, Tempol (Kang, Ma et al. 2009). Here we show that PVN specific NFκB blockade reduced the PIC and ROS reactions typically associated with AngII-induced hypertension, highlighting the central position that PVN NFκB plays in regulating the neurogenic component of the hypertensive response.

Symapthoexcitation is a major component of hypertension, and increased levels of AngII in the CNS can enhance sympathetic outflow through ROS and AT_1R activation and a subsequent down-regulation of nNOS (Zanzinger, Czachurski et al. 1995; Liu, Murakami et al. 1998; Campese, Shaohua et al. 2005; Gao, Wang et al. 2005; Guggilam, Cardinale et al. 2011), and the PVN plays a role in modulating sympathetic outflow (Allen 2002). NO, a well known sympatho-inhibitory neurotransmitter, when blocked, results in elevated MAP and sympathoexcitation (Campese, Ye et al. 2002; Campese, Shaohua et al. 2005). The expression and presence of nNOS, an indirect indicator of downstream sympathetic activity, is inversely proportional to the level of sympathetic outflow (Wang, Liu et al. 2005; Li, Wang et al. 2006; Guggilam, Patel et al. 2008). Furthermore, increased superoxide can interact with the nNOS-produced NO, forming $OONO^{\bullet}$ and further affecting the decrease in NO bioavailability, also resulting in enhanced sympathoexcitation (Zanzinger 2002; Yu, Zhang et al. 2010). Recently, Ang(1-7) and MasR activation have been shown to increase nNOS activity and NO release (Sampaio, Souza dos Santos et al. 2007). The current study shows that following AngII treatment, nNOS is decreased within the PVN, potentially indicating an increased sympathetic response. Also in the present study, $OONO^{\bullet}$ was increased in the AngII-treated rats, which, following bilateral NFκB inhibitor administration, was decreased. The $OONO^{\bullet}$ decrease paralleled that of $O_2^{\bullet-}$ and was concurrent with the nNOS increase, indicating that NFκB plays potentially a deciding role in increasing NO availability and regulating sympathetic outflow. Therefore, bilateral NFκB inhibition increased nNOS and reduced $O_2^{\bullet-}$ and $OONO^{\bullet}$, potentially through protective RAS component balance, thus increasing nNOS and the capacity for NO availability and potentially decreasing subsequent sympathetic outflow.

In conclusion, this study shows that following the activation of the pro-hypertensive arm of the RAS, PICs are increased, both of which can act to increase the activity of NFκB, leading to the transcription of additional modulators of the hypertensive state in a positive feed-forward manner. NFκB acts further to increase, along with AngII, the presence of ROS, such as $O_2^{\bullet-}$, which subsequently affects the present NO levels, thereby increasing sympathoexcitation. Blockade of NFκB at two separate locations in its activation pathway prevents these changes, restoring balance and promoting the anti-hypertensive arm of the RAS, including ACE2 and the MasR, as well as reducing PIC and ROS expression and elevating nNOS, all of which contributes to a reduction in MAP and an improvement in the AngII-induced hypertensive state. However, this signaling mechanism must be further studied to delineate the manner by which NFκB and $O_2^{\bullet-}$ interact within the PVN in AngII-induced hypertension. We propose that AngII activation of NFκB increases PICs and $O_2^{\bullet-}$, tipping the balance of the RAS in favor of the pro-hypertensive arm and decreasing the anti-hypertensive arm, resulting in a further increase in PIC and ROS expression, in a vicious positive feed-forward mechanism (Figure 5.9). Limitations for this study include the use of the AngII-induced hypertensive model, since this does not represent all modes of hypertension. Furthermore, we only explored the PVN region in this hypertensive model. There are multiple cardio-relevant regions of the brain that can play a role in modulating the hypertensive response; however, we feel that the PVN is of importance due to its integrative functions. Moreover, as the literature suggests, Adenoviruses are well known for their lack of cell specificity and can be expressed by neurons and glia as well as participate in retrograde transport (Sinnayah, Lindley et al. 2002). For this reason, we employed NFκB decoy oligodeoxynucleotides to further verify our results. Finally, to show the minimal effects of the potential perfusion of bilateral PVN NFκB decoy infusion leakage into the third ventricle and its

subsequent effect on other regions of the brain, ICV infusion of the same dose at the same infusion rate could have been performed, but we feel, based on similar experiments, that though third ventricle leakage may have occurred, its effects would be negligible. In the current clinical environment where novel hypertensive therapeutic measures are being sought, this study provides a conceptual basis for including NFκB inhibitors that can specifically act within the brain as a possible future pharmacological approach for the treatment of hypertension.

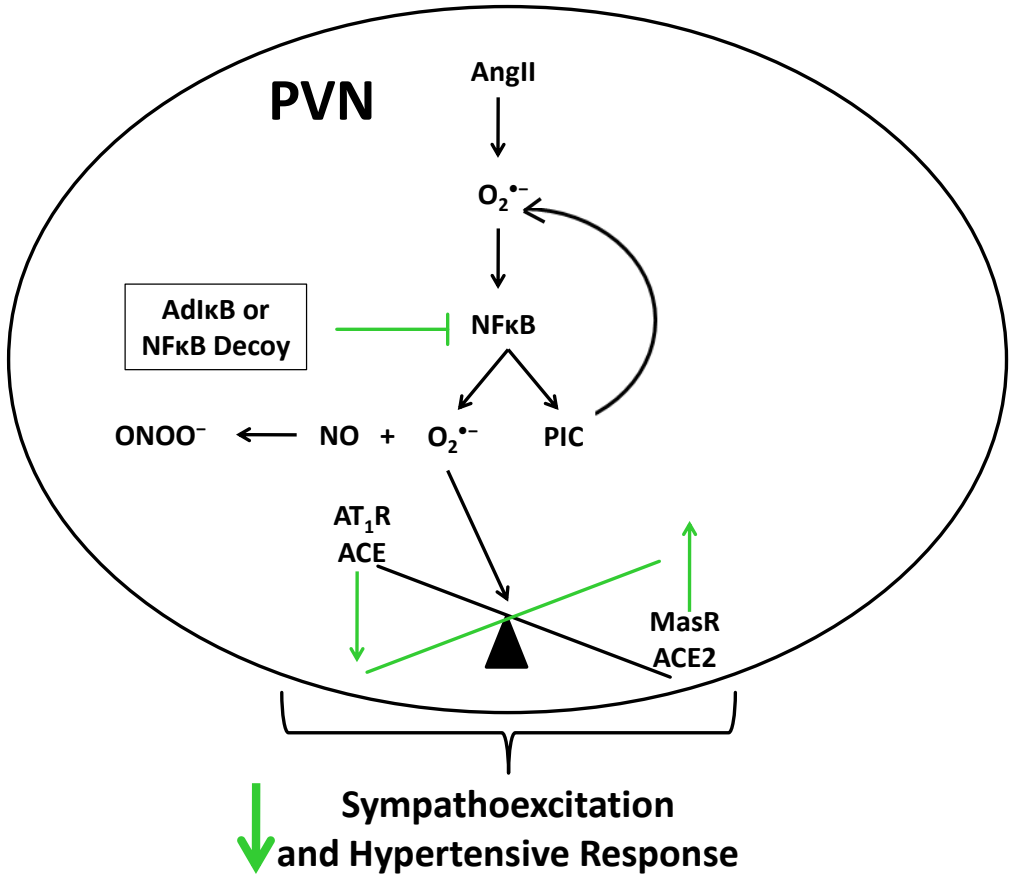

Figure 5.9. Schematic showing the proposed signaling mechanism in the PVN. Ang II leads to an exasperated hypertensive response via NFκB and modulation of the balance between the pro- and anti-hypertensive arms of the renin-angiotensin system (solid lines/arrows) and how NFκB inhibition attenuates this response (green lines/arrows). Ang II activates NFκB via an AT_1R/superoxide pathway. NFκB increases transcription of PICs and ROS production genes, as well as the pro-hypertensive RAS components, while decreasing the anti-hypertensive RAS components. The increased PICs and ROS positively feeds back upon NFκB (while also signaling for increased sympathetic outflow). These signals combine to propagate the hypertensive state.

PERSPECTIVES

Increasing evidence indicates that central nervous system mechanisms play an important role in the pathogenesis of cardiovascular disease. In this study, we demonstrate that inflammatory molecules, specifically the transcription factor NFκB, within the PVN, can modulate the hypertensive response. Additionally, we demonstrate that inflammation is a double-edged weapon that not only up-regulates the pro-hypertensive axis of the RAS, but also down-regulates the protective anti-hypertensive axis of the RAS. Thus, inflammation-mediated modulation of the RAS system within the brain might be an important critical contributor to neurogenic hypertension. Since inflammation and RAS are potent inducers of oxidative stress and NFκB has been shown to respond to and induce oxidative stress, it would be advantageous to target NFκB to better treat neurogenic hypertension. While the current methods used within this study are impractical for current clinical administration, one can explore the use of NFκB small molecule inhibitors that might cross the BBB, thereby targeting the brain's source of inflammation and oxidative stress for controlling and treating this debilitating condition.

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CHAPTER 6

CONCLUDING REMARKS

OVERALL SUMMARY OF FINDINGS

Hypertension is one of the world's great public health problems, affects more than 74 million American adults, and is the leading cause of death worldwide (2002; Lloyd-Jones, Adams et al. 2010). Hypertension is not only a serious condition with its own high morbidity and mortality rates, it is also a major risk factor for multiple cardiovascular diseases (CVDs), including myocardial infarction, stroke, heart failure, atrial fibrillation and peripheral arterial disease. Moreover, with the rise in additional causes for increased blood pressure, including obesity, excessive salt intake, poor lifestyle choices and a growing population of aging individuals, the incidence of hypertension is rising (Lloyd-Jones, Adams et al. 2010). While the condition is easily detectable, it still remains poorly controlled in more than half of identified hypertensive patients. Current therapeutics for primary hypertension are multiple and varied, targeting and exploiting the many endogenous controlling mechanisms of normal pressure response as well as pathophysiological contributors towards hypertension (Izzo, Sica et al. 2008). However, many of these therapies fail to have a beneficial effect, prompting the need for novel therapeutic approaches and a greater understanding of the mechanistic pathways involved in the pathogenesis and maintenance of hypertension.

Recent evidence suggests that hypertension is a chronic low-grade inflammatory condition where pro-inflammatory cytokines (PICs), such as tumor necrosis factor-*alpha* (TNF), interleukin (IL)-1β and IL-6, have emerged as major contributing factors in the pathogenesis of hypertension and other CVDs (Mann 2002; Ruiz-Ortega, Ruperez et al. 2002; Ferrario and Strawn 2006; Sriramula, Haque et al. 2008; Kang, Ma et al. 2009; Cardinale, Sriramula et al. 2010). The renin-angiotensin system (RAS), especially the effector peptide angiotensin II (Ang II), not only modulates the hypertensive response, but also induces the inflammatory response

associated with hypertension (Kalra, Sivasubramanian et al. 2002). Moreover, this inflammatory response is capable of regulating local and systemic RAS components, including increasing the pro-hypertensive axis (Ang II, angiotensin converting enzyme (ACE) and Ang II type-1 receptor (AT_1R)) while silencing those of the anti-hypertensive axis (ACE2, AT_2R, Ang (1-7) and the Mas receptor). The Ang II-mediated inflammatory response appears to occur through several mechanisms, including activation of monocytes and macrophages, as well as the production of adhesion molecules, chemokines, PICs and reactive oxygen species (ROS) (Arenas, Xu et al. 2004; Shi, Diez-Freire et al. 2010). While still not fully known, the interaction of these various hypertensive regulatory pathways are becoming increasingly important to understand, especially in regards to their contribution towards the pathogenesis of hypertension. Therefore, elucidating the mechanism by which these signaling pathways interact is critical for the development of novel therapeutics in the fight to control the debilitating effects of hypertension.

Chapter 2 acknowledges evidence that the RAS and Ang II can increase the production of PICs and contribute to the hypertensive state, and that blocking the PIC TNF can prevent some of the deleterious changes associated with hypertension and the ACE/Ang II/AT_1R RAS axis (Funakoshi, Ichiki et al. 1999; Kalra, Sivasubramanian et al. 2002; Muller, Shagdarsuren et al. 2002; Ruiz-Ortega, Ruperez et al. 2002; Elmarakby, Quigley et al. 2006). The suggested mechanism potentially involved a positive feed-forward system whereby Ang II increases TNF and ROS, which in turn further increases injurious ACE/Ang II/AT_1R RAS axis components, and decreases protective ACE2/Ang (1-7)/Mas RAS components. Therefore, we aimed to determine if administration of TNF alone could regulate components of the RAS, and if so, was this mechanism achieved through modulation of ROS. TNF infusion for five days increased the components of the deleterious axis of the RAS, including angiotensinogen, ACE, Ang II and the

AT_1R. TNF infusion also increased gp91*phox* (the NADPH oxidase catalytic subunit), total ROS and superoxide, and decreased the protective RAS components ACE2 and the Mas receptor. These changes were reversed following administration of the superoxide dismutase (SOD) mimetic, Tempol, or the TNF receptor mimetic, etanercept, indicating that the TNF-induced TNF-driven pathway required ROS, especially superoxide, for dysregulation of the individual RAS arms and propagation of the hypertensive state. In conjunction with the understanding that Ang II increases TNF and that use of PIC and ROS inhibitors can reduce the hypertensive response, we demonstrate here that the ROS appears to evolve through a TNF-activated mechanism, therefore highlighting the Ang II/TNF/ROS pathway. This pathway in turn reduces the protective RAS axis (ACE2/Ang (1-7)/Mas) while up-regulating the more injurious aspects of the pro-hypertensive RAS arm (ACE/Ang II/AT_1R). This study underscores the role that TNF plays in the ROS-induced dysregulation of the RAS and the ROS-driven mechanism behind TNF-induced cardiac dysfunction, further spotlighting the role that PICs such as TNF may play in the differential RAS regulation in the pathogenesis of the hypertension.

Chapter 3 discusses the interaction between the RAS, PICs and ROS demonstrated by work in our laboratory and others (Brasier, Li et al. 1996; Sasamura, Nakazato et al. 1997; Arenas, Xu et al. 2004; Sriramula, Haque et al. 2008). Here we showed that untreated spontaneously hypertensive (SHR) rats have increased AT_1R expression, an important component of the pro-hypertensive portion of the RAS. Histone Deacetylase inhibition (HDACi) through valproic acid (VPA) treatment attenuated this increase and the increased production of PICs. Reports previously indicated that Ang II is controlled by, and controls, HDAC-induced changes in gene and protein response (Kee, Sohn et al. 2006; Lu and Yang 2009). Here we show a possible new mechanism involving the regulation of Ang II responses as directed through the

AT_1R. HDACi reduces AT_1R gene expression and receptor density, thereby ameliorating the actions of Ang II. This alteration in AT_1R expression could be either through direct HDACi effects on the receptor's production and function, or through the effects of HDACi on inflammatory gene response during hypertension. Thus, results of this study showed that long-term HDACi through VPA attenuated mean arterial pressure (MAP) and cardiac hypertrophy, possibly through modulation of atrial natriuretic peptide (ANP), Collagen IV and AT_1R. HDAC inhibition also attenuated the increased inflammatory response, including TNF and nuclear factor-*kappa*B (NFκB), as well as the increase in ROS and gp91*phox*. These findings suggest that HDACi with VPA reduced inflammation, ROS, and AT_1R, thereby attenuating hypertension and its secondary consequences in SHR rats.

In Chapter 4, we demonstrated that chronic Ang II infusion resulted in cardiac hypertrophy and hypertension, increased PIC and gp91*phox*, and decreased expression of neuronal Nitric Oxide Synthase (nNOS) within the hypothalamic paraventricular nucleus (PVN). Perhaps more importantly, Ang II-infused rats had an increased expression of the injurious pro-hypertensive RAS components ACE and AT_1R, and a decreased expression of the protective anti-hypertensive RAS components ACE2, AT_2R and the Mas receptor. Central blockade of TNF with etanercept resulted in attenuation of hypertension, cardiac hypertrophy and PIC expression, decreased oxidative stress, as well as a restored balance between the protective and deleterious RAS arms within the hypothalamic PVN. The beneficial effects of central TNF blockade in Ang II-induced hypertensive responses appear to be mediated by the returned balance of the central RAS, especially within the PVN. Our findings provide further evidence for the involvement of the RAS within the PVN and its interaction and mediation through TNF in the neurogenic component of hypertension.

Finally, in Chapter 5, we demonstrated that following the activation of the pro-hypertensive arm of the RAS, PICs are increased, both of which can act to increase the activity of NFκB within the PVN. This in turn leads to the transcription of additional modulators of the hypertensive state in a positive feed-forward manner that contributes to the hypertensive state. Furthermore, NFκB acted to increase, along with AngII, the presence of ROS, such as superoxide, which subsequently decreased the presence of Nitric Oxide (NO), thereby increasing sympathoexcitation. Blockade of NFκB within the PVN at two separate locations in its activation pathway, prior to entering the nucleus (via PVN transfection with an Adenoviral vector containing Inhibitory-*kappa*B), and after nuclear translocation (via PVN infusion of NFκB decoy oligodeoxynucleotide), prevented these changes. These treatments promoted the anti-hypertensive arm of the RAS, including ACE2 and the Mas receptor, as well as reducing PIC and ROS expression, and elevating nNOS, all of which contributes to a reduction in MAP and an improvement in the AngII-induced hypertensive state. Therefore, we proposed that AngII activation of NFκB within the PVN increases PICs and superoxide, tipping the balance of the RAS in favor of the pro-hypertensive arm and decreasing the anti-hypertensive arm, resulting in an even further increase in PIC and ROS expression, resulting in a vicious and positive feed-forward mechanism.

SIGNIFICANCE OF RESEARCH AND FUTURE DIRECTIONS

Current treatment regimens for hypertension are only partially effective in controlling the disease state. These therapeutics act on the endogenous mechanisms that control pressure response in normal and pathological conditions. Briefly, these therapies can include lifestyle modifications and/or a multitude of pharmacological therapeutics. Lifestyle modifications can include weight reduction, sodium reduction, diet modification, increased physical activity and

moderation of alcohol and tobacco consumption, amongst others. Common pharmacological interventions can include any number of drugs alone or in combination, including, but not limited to, diuretics, β-blockers, α_1-blockers, Ca^{2+} channel blockers, ACE inhibitors and angiotensin receptor blockers (ARBs) (Izzo, Sica et al. 2008). However, while many current therapeutics have clearly reduced the morbidity and mortality of some hypertensive patients, they still fail to have a beneficial effect on over 50% of treated individuals, prompting the need for novel therapeutic approaches and a greater understanding of the mechanistic pathways involved in the pathogenesis and maintenance of hypertension.

The complexity and somewhat unclear signaling mechanisms involved in hypertension are of intense investigational interest. Recent evidence shows that hypertension is a chronic low-grade inflammatory condition (Ferrario and Strawn 2006; Sriramula, Haque et al. 2008; Kang, Ma et al. 2009; Cardinale, Sriramula et al. 2010) where PICs, such as TNF, have emerged as major contributing factors in the pathogenesis of hypertension (Mann 2002; Ruiz-Ortega, Ruperez et al. 2002; Sriramula, Haque et al. 2008; Kang, Ma et al. 2009). Despite the overwhelming evidence linking TNF to CVDs, large clinical trials targeted against this PIC have been largely unsuccessful (RENAISSANCE, RECOVER and ATTACH) (Anker and Coats 2002; Mann, McMurray et al. 2004); it is speculated that the lack of success of these trials lies not in the use of anti-cytokine agents, but the use of system wide TNF inhibition and the consequences resultant from this type of therapy (Mann, McMurray et al. 2004). Therefore, the need for improved knowledge of the contributing pathways to hypertension, and the requirement for continued therapeutic developments remain of the utmost importance. The studies conducted herein provide significant advancements in delineating mechanistic pathways and potential therapeutic targets for the treatment of hypertension.

The studies described here underscore the role that inflammation plays in hypertension and the means by which it is regulated, providing a strong foundation for the future development of novel therapeutic options. It shows that TNF plays a major part in the ROS-induced dysregulation of the RAS and the ROS-driven mechanism behind TNF-induced cardiac dysfunction. These studies also show how central blockade of TNF with etanercept can attenuate hypertension, cardiac hypertrophy and PIC expression, decrease oxidative stress, and restore the balance between the protective and deleterious RAS arms, within the hypothalamic PVN, pointing to a role for TNF in the neurogenic component of hypertension as well. Furthermore, we proposed that AngII activation of NFκB within the PVN increases PICs and superoxide, tipping the balance of the RAS in favor of the pro-hypertensive arm and decreasing the anti-hypertensive arm, resulting in sustained hypertension. This study showed that specifically targeting NFκB within the PVN can attenuate the hypertensive state, providing a conceptual basis for including NFκB inhibitors that can specifically act within the brain as a possible future pharmacological approach for the treatment of hypertension. Finally, we demonstrated how the use of VPA, an anti-seizure and bipolar drug currently used in clinical settings, administered long-term without any adverse effects towards the treated animals, was successful in the treatment of hypertension and its consequences, including cardiac hypertrophy, systemic inflammation and end organ damage due to ROS. This study outlines the importance of the continuous drug administration necessary to treat hypertension and also provides sufficient evidence for the use of HDACi to reduce not only blood pressure, but cardiac hypertrophy and the inflammatory state associated with hypertension. Whereas ACEi and ARBs only target a portion of the systemically activated RAS, combined, these studies identify the involvement of several inflammatory mediators (TNF, NFκB and HDAC), through ROS, in activating and

dysregulating the local RAS within the heart and brain and contributing to the pathogenesis and sustainment of hypertension.

Although we believe that these studies identify novel pathways through which TNF, NFκB, ROS and the RAS interact in the heart and brain in hypertension, further studies are required to understand the exact mechanistic pathways involved, as well as identify additional possible therapeutic targets whose inhibition/activation would not lead to adverse responses (as observed in several clinical trials against TNF). Further identification of additional mediators of TNF and NFκB, determining the role of additional cardio-regulatory centers in the brain in the hypertensive response, and elucidating how peripheral modulators signal to the brain, would all greatly improve this knowledge base. Moreover, a closer look at the protective axis of the RAS provides an appealing option for the future of therapeutics. Based on these studies, a clear link between inflammatory mediators and the dysregulation of the RAS is clear, but more work must be done to develop safe and effective means by which to positively influence this mechanism. Other issues also present both challenges and avenues of exploration for future hypertensive studies. For instance, specific Sirtuins and hypoxia inducible factors (HIF) are known to interact with NFκB and differentially regulate its actions, but how and in what pathological states this occurs is unknown (Biala, Tauriainen et al. ; Miyazaki, Ichiki et al. 2008). Also, thus far, our labs interest has been with the role of inflammation in the PVN, but attention must be made for the rostral ventrolateral medulla (RVLM) and supraoptic nucleus (SON), amongst others, to determine the signaling patterns between these regions and to the periphery and determine how this signaling can be subjected to controlling in hypertension. Finally, speculations of the manner by which peripheral signals interact with the brain are prevalent, but identifying this with more certainty can ultimately lead to the development of better, more efficient therapeutics. All

together, the data presented here, along with the contributions of current and future studies, will go a long way in aiding in the identification of efficacious treatments in the constant pursuit to alleviate patients suffering from the debilitating effects of hypertension.

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APPENDIX

LETTER OF PERMISSION

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VITA

Jeffrey Paul Cardinale was born in Bedico, Louisiana, on October 11th, 1983, to Gerald and Yvette Cardinale. He attended Saint Paul's School in Covington, Louisiana, and graduated in the top 10% of his class in May, 2002. Following high school, he attended Louisiana State University in Baton Rouge, Louisiana, where he gained an appreciation and fostered an interest in medicine and the basic sciences while working in the clinics and labs at the Louisiana State University School of Veterinary Medicine, Department of Veterinary Clinical Sciences. Through the mentorship and guidance of Susan Eades, D.V.M., Ph.D., Diplomate ACVIMLA, and following his graduation in May, 2006 with a Bachelor of Science degree, he was introduced to the work of Associate Professor Joseph Francis, B.V.Sc. & A.H., M.V.Sc., Ph.D., in cardiovascular pathophysiology, in whose lab he began the pursuit of his doctoral degree in August, 2006. During his time, he gained a working understanding of the basic physiological and pathophysiological mechanisms of the heart and brain in cardiovascular disease. During these studies, in October, 2008, he married Lauren Elise Mesman. Following his graduation with his Doctor of Philosophy degree (Ph.D.) in August, 2011, he will continue his studies at Louisiana State University Health Sciences Center in New Orleans, Louisiana, to pursue his medical doctorate degree. His career goals are to combine his knowledge of the basic sciences with his future studies in medicine in the hopes of conducting clinical based research in cardiovascular diseases.